ABSTRACT BOOK

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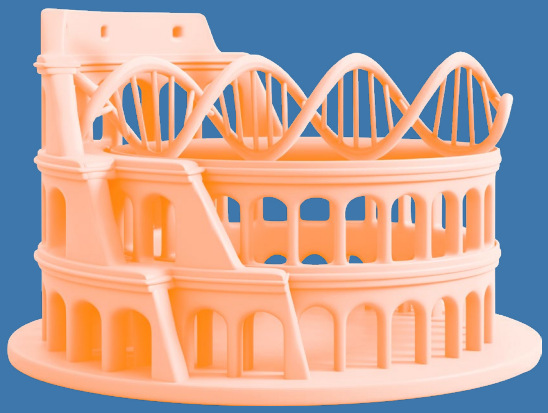

EUROPEAN SOCIETY OF GENE & CELL THERAPY

Società Italiana di Terapia Genica e Cellulare

Invited Speakers

CTC03

Regulatory non-clinical aspects of gene-editing based products

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With the rising number of clinical trial applications involving gene-editing based products, non-clinical programs are evolving addressing the efficiency and safety of various gene-editing approaches. Editing of human cells based on the non-homologous end joining repair mechanism of induced double strand breaks and subsequent infusion of these cells still represents the predominant gene-editing approach that enters clinical development.

This presentation will outline the regulatory non-clinical requirements needed for translating such products into the clinic. Particular emphasis will be placed on the methodology including in silico and in vitro methods and the extent of non-clinical data needed to assess both on-target and off-target gene editing.

INV02

AAV1-hOTOF Gene Therapy for Autosomal Recessive Deafness 9

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Background: Mutations of *OTOF* gene can cause autosomal recessive deafness 9 (DFNB9), which is characterized by congenital or prelingual, severe-to-complete bilateral hearing loss. However, there is no drug available for hereditary deafness. Here, we report the single-arm trial investigating the safety and efficacy of gene therapy for children with DFNB9 via unilateral or binaural administration of AAV1-hOTOF: adeno-associated virus (AAV) serotype 1 carrying a human *OTOF* transgene.

Methods: Patients (1-18 years of age) with severe-to-complete hearing loss were screened and enrolled. AAV1-hOTOF was injected into unilateral or binaural ear(s) through round window. The

primary outcome was dose-limiting toxicity. The secondary outcome included safety (adverse events) and efficacy (auditory function and speech perception).

Results: 11 patients were enrolled. All patients had average auditory brainstem response baseline of >95 dB. Follow-up ranged from 13 to 52 weeks. No dose-limiting toxicity or serious adverse event occurred. Ten children had hearing recovery. In the patient receiving 9×10^{11} vg dose unilaterally (patient #1), the average ABR threshold was recovered to 68 dB at 4 weeks, 53 dB at 13 weeks, 45 dB at 26 weeks and 28 dB at 52 weeks. In patients who received 1.5×10^{12} vg dose unilaterally (patients #2-6), the average ABR thresholds were >95, 45, 45, 30, and 50 dB at 52 weeks, respectively. In patients with binaural administration of 1.5×10^{12} vg dose (patients #7-11), the average ABR thresholds of left (right) ear were also reduced within 26 weeks of follow-up. The hearing recovery showed time-dependent. Speech perception was improved in patients who had hearing recovery. The ability of sound source localization was recovered in patients with binaural administration.

Conclusions: The AAV1-hOTOF gene therapy is safe and efficacious as a novel treatment for patients with DFNB9.

INV03

Exagamglogene Autotemcel For Transfusion-Dependent β-Thalassemia

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Exagamglogene autotemcel (exa-cel) is a non-viral cell therapy that reactivates fetal hemoglobin (HbF) via *ex vivo* CRISPR-Cas9 gene-editing of autologous CD34+ hematopoietic stem and progenitor cells (HSPCs). Exa-cel is approved as a one-time treatment for patients (pts) with transfusion-dependent β-thalassemia (TDT) based on positive results from a pre-specified interim analysis of a 24-month (mo), phase 3 trial of exa-cel in pts age 12-35y with TDT and a history of ≥100 mL/kg/y or ≥10 U/y packed red blood cell (RBC) transfusions in the 2y before screening. Enrollment and dosing are complete; trial is ongoing. Primary endpoint is transfusion independence defined as proportion of pts maintaining a weighted average hemoglobin (Hb) ≥9 g/dL without RBC transfusion for ≥12 consecutive mos (TI12). Key secondary endpoint is

proportion of pts maintaining a weighted average Hb ≥9 g/dL without RBC transfusion for ≥6 consecutive mos (TI6). Evaluable pts must have ≥16 mos of follow-up after exa-cel infusion. Evaluation of TI12 and TI6 started 60 days after last RBC transfusion for post-transplant support or TDT management. Pts completing trial enrolled in long-term follow-up Study 131. As of 19 March 2024, 56 pts (mean age 21.2 [range 12-35]y; 20 [35.7%] age ≥12 to <18y; 35 [62.5%] with severe genotypes [β^0/β^0 or β^0/β^0-like], median annualized transfusion volume 206.7 mL/kg) received exa-cel; median follow-up 33.3 (range 3.1-62.3) mos. Of these, 43 pts completed 2 yrs of follow-up in CLIMB-THAL-111 and enrolled in Study 131. Following infusion, all pts engrafted neutrophils and platelets (median 29 and 43.5 days, respectively). Of the 52 pts evaluable for primary and key secondary endpoints, 49 (94.2%) achieved TI12 and TI6 (95% CI: 84.1%, 98.9%). Pts achieving TI12 stopped transfusions 32.7 (SD, 17.3) days after exa-cel infusion and remained transfusion independent for 31.0 (range, 12.8, 59.4) mos. For the 3 pts not achieving TI12, two stopped transfusions and achieved TI12 in Study 131 and one had been transfusion free for 10.6 months before a transient episode of anemia related to viral gastroenteritis (since then has been transfusion free for 3.4 months). For all pts, mean total Hb was 11.6 g/dL at Month 3 (≥12g/dL Month 6 onward) and HbF was 8.0 g/dL at Month 3 (≥ 11 g/dL Month 6 onward) with pancellular distribution (≥95% RBCs expressing HbF Month 6 onward). Proportion of edited *BCL11A* alleles was stable over time in bone marrow $CD34^+$ and peripheral blood nucleated cells. Pts not yet evaluable and with sufficient follow-up were also transfusion-free. All pts had ≥1 adverse event (AE), most were Grade 1 or 2; 50 (89.3%) pts had AEs of Grade 3 or 4 severity. Most common AEs were febrile neutropenia (60.7%), headache (55.4%), and stomatitis (53.6%). As previously reported, 2 pts (3.7%) had SAEs considered related to exa-cel which all resolved. There were no deaths, discontinuations, or malignancies. Safety profile of exa-cel remains generally consistent with myeloablative busulfan conditioning and autologous transplantation. These results confirm the potential for exa-cel to provide a one-time functional cure to pts with TDT.

INV04

Developing single-course gene editing medicines to treat cardiovascular disease

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1: Verve Therapeutics

Background: Cumulative lifelong exposure to high levels of blood cholesterol carried in lipoproteins drives the development and progression of atherosclerotic cardiovascular disease (ASCVD), the global leading cause of mortality. For people who have ASCVD, or are at risk for developing it, it is critical to maintain blood low density lipoprotein cholesterol (LDL-C) at target levels for as long as possible. However, under the chronic care model of injectable and oral therapies that require lifelong adherence, few patients achieve LDL-C targets and even fewer maintain target levels over years to decades. The unmet need for cholesterol-lowering is especially pronounced in subsets of the population with genetic and refractory hypercholesterolemia syndromes. Gene editing medicines have the potential to overcome the limitations of the chronic care model and deliver lifelong LDL-C lowering after a single-course of treatment. Here we describe an *in vivo* base editing approach to inactivate the *PCSK9* gene, a target that is genetically and pharmacologically validated to lower LDL-C and reduce cardiovascular risk.

Methods: The *PCSK9* program consists of two separate product candidates that utilize lipid nanoparticles (LNPs) for *in vivo* hepatocyte delivery of an mRNA encoding an adenine base editor (ABE) and gRNA. Both candidates inactivate *PCSK9* by introducing a single A-to-G base pair

change without a double-strand break. The candidates have the same ABE and gRNA but differ in their LNP delivery systems. The first product candidate (VERVE-101) relies primarily on endogenous LDL receptor- (LDLR-) mediated endocytosis for hepatocyte delivery. The LNP for the second product candidate (VERVE-102) consists of a different lipid formulation and carries a GalNAc targeting ligand that enables endocytosis via the asialoglycoprotein receptor in addition to LDLR. The addition of GalNAc as a second method for hepatocyte uptake may enable more robust delivery, especially in the setting of LDLR deficiency seen with familial hypercholesterolemia. First-in-human clinical trials have been initiated for both programs in patient populations with high-risk cardiovascular disease.

Results: VERVE-101 led to dose-dependent reductions in PCSK9 and LDL-C in a phase 1b clinical trial that represented the first proof-of-concept for *in vivo* base editing in humans. Transient laboratory abnormalities in one participant, suspected to be due to the VERVE-101 LNP, led to a pause in enrollment pending investigation. The single-ascending dose phase 1b Heart-2 trial of VERVE-102 using the GalNAc-LNP is ongoing.

Conclusions: ASCVD is the leading cause of mortality and the requirement for indefinite adherence to chronic care therapies contributes to poor real-world LDL-C control. Gene editing is an investigational approach that has the potential to permanently inactivate cholesterol-raising genes in the liver, and first-in-human proof-of-concept for LDL-C lowering has been demonstrated. Additional clinical investigation of gene editing is warranted to determine if it can address the persistent unmet need in ASCVD.

INV06

Safety Profile of Recombinant AAV Vectors in Nonhuman Primates and Humans: Bench to Bedside and Back

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Recombinant adeno-associated viruses (AAV) are the current vector of choice for locally or systemically administered gene therapies targeting the central nervous system, skeletal muscles, or heart. Many programs using high doses of intravenous AAV have faced test-article-related serious adverse events that were often not predicted by preclinical safety studies. On the other hand, class-effect toxicities that are highly prevalent in preclinical research - such as AAV-related dorsal root ganglia pathology - have rarely translated to serious adverse events in clinical trials. In this presentation, we will review meta-analysis data including nonhuman primates (NHPs) that received intrathecal or intravenous AAV at the Gene Therapy Program. We will review well-known class-effect toxicities of AAV and how they present in NHPs and consider their relevance to humans as well as discuss how to approach a benefit-risk-based philosophy when faced with test-article-related toxicities in preclinical studies.

INV07

Targeted viral vectors for in vivo gene therapy

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Millions of patients worldwide suffer from various hematological diseases, malignancies, and disorders such as HIV, Sickle Cell Disease, or Fanconi Anemia either receiving no treatment or taking life-long non-curative medication. Hematopoietic stem cell (HSC) gene therapy is a highly promising and permanently curative treatment option as shown by the numerous successful clinical trials and FDA approvals in the past years. However, approaches currently rely on the modification of HSCs outside the patient's body (*ex vivo*) which requires highly specialized cleanroom facilities as and personnel severely impacting the feasibility as well as geographically limiting the accessibility to this life-saving treatment.

To overcome these logistical as well as financial bottlenecks, many research groups, startups, and established biotech companies currently focus on the development of novel strategies to perform HSC gene therapy directly in the patient *(in vivo*) with an intravenous administration of the required agents. Although simple in theory, the administration of especially lentiviral vectors *in vivo* is very challenging due to the adaptive and innate immune system effectively surveilling the blood and inactivating viral and virus-like particles, serum inactivation through the complement system, and unwanted uptake of viral particles into non-target cells through the readily expressed viral receptors.

To systematically address these limitations and develop portable in vivo HSC gene therapy applications, we combined discoveries in viral envelope/particle surface-engineering with our recently reported HSC-targeting technology to effectively and specifically deliver transgenes as well as nucleases into human HSCs *ex vivo* and *in vivo*. These HSC-targeted viral vectors have the potential to seek out HSCs *in vivo*, reduce the off-target risk, and most importantly, overcome current accessibility issues democratizing HSC gene therapy.

INV08

A first in human clinical study using lentiviral vector transduced CD34+ cells encoding a novel FVIII transgene for the treatment of hemophilia A

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Although there is now an FDA approved AAV-based gene therapy treatment for hemophilia A, there remain major concerns, for example i) pre-existing anti-AAV or inhibitory anti-FVIII antibodies, ii) unsustained FVIII expression, and iii) the inability to treat young children with developing livers. In contrast, we developed a lentiviral-based approach targeting CD34 hematopoietic stem cells (HSC). The FVIII encoding transgene was engineered for high level FVIII expression (termed ET3), which is partially based on the high expression levels achieved using porcine FVIII sequences and is B-domain deleted. The LV-vector is self-inactivating with a CD68

promoter, which we have shown restricts expression to the myeloid lineage, and is termed CD68-ET3-LV. Our first in human clinical trial utilized mobilized autologous CD34+ cells transduced ex-vivo with CD68-ET3-LV. Five subjects were transplanted with CD68-ET3-LV transduced cells after conditioning with treosulfan and fludarabiine. To date, the annual bleeding rate is zero for all participants, FVIII activity levels have been stable for over 2 yrs, and FVIII activity is in the normal range for two subjects. This clinical trial demonstrates clinically significant, and stable, FVIII expression levels with protection from bleeding, and it demonstrates the feasibility for using LV-transduced autologous HSC gene therapy for hemophilia A.

INV10

mRNA trans-splicing dual AAV vectors for treatment of Stargardt disease and Usher syndrome type 1B

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Adeno-associated viruses are the gold standard for gene transfer in gene therapies. An important limitation of these vectors is their limited DNA packaging capacity, which complicates the therapy of large genes. To this end, various dual AAV vector systems have been developed in the past, which have some drawbacks in terms of safety, flexibility or efficiency. In a recent study, we have developed an efficient and flexible dual AAV vector method based on reconstitution via mRNA trans-splicing (REVeRT). We have used this technology for gene replacement therapy of two genes associated with hereditary retinal dystrophies: i) MYO7A, whose defects lead to Usher syndrome - the most common genetic form of deafblindness. ii) ABCA4, whose defects lead to Stargardt disease. REVeRT achieves efficient expression of MYO7A and ABCA4 in mice, pigs and human retinal organoids at the transcript and protein level. To test the efficiency of MYO7A supplementation at the cellular level, we have developed a novel human retinal organoid model that reliably exhibits some features of Usher syndrome. In addition, preclinical testing of ABCA4 supplementation in Abca4-deficient mice and human retinal organoids has been successfully conducted, laying the foundation for clinical trials in the near future.

INV11

TCR-engineered T cell therapy comes of age: generating safe and effective treatments for acute myeloid leukemia

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Background. T-cell receptor (TCR)-engineered T cells may offer a unique therapeutic opportunity for highly heterogeneous diseases with low mutational burden, such as acute myeloid leukemia (AML), thanks to their ability to recognize every tumor antigen with an exquisite sensitivity. Here, we isolated and functionally characterized a peculiar TCR specific for Cathepsin G (CG), a serine protease normally present in neutrophil azurophilic granules and overexpressed by AML blasts/leukemic stem cells.

Methods. Anti-tumoral lymphocytes were expanded from peripheral blood mononuclear cells of two healthy donors through repeated stimulations with autologous antigen presenting cells pulsed with overlapping pentadecapeptides covering the entire CG protein. Bulk TCR sequencing was employed to identify and pair tumor-specific αβ clonotypes. Redirection of T-cell specificity was achieved through the TCR gene editing approach.

Results. We isolated 3 different CG-specific TCRs recognizing peptides restricted by 4 different HLA alleles. Of interest, p56-TCR proved able to recognize 2 different CG peptides stemming from the same pentadecapeptide when presented on HLA-A*24:02 and HLA-C*07:02, that altogether are expressed by approximately 35% of the Caucasian population. Results of in silico prediction and in vitro validation clearly highlighted the HLA-A*24:02-restricted nonamer recognized by the TCR. Instead, for the HLA-C*07:02 allele we could not identify any shorter peptide recognized with higher efficacy than the 15-mer. Natural peptide processing and presentation was confirmed by the ability of p56-TCR edited T-cells to specifically eliminate in vitro and in vivo primary leukemic blasts expressing either HLA-A*24:02 or HLA-C*07:02. Noticeably, in vivo results highlighted a statistically significant difference in leukemia burden in mice treated with CG-specific T-cells versus mice infused only with HLA-A*24:02+ or HLA-C*07:02+ leukemic cells ($p<0.0001$) and the eradication of the disease in the bone marrow and in the spleen. Furthermore, the results of alanine scanning and colony-forming unit assays revealed no evidence of on-target/off-tumor and off-target/off-tumor toxicities, underlining the safety profile of the engineered T cells.

Conclusions. CG-engineered T cells can be effectively used to target CG-expressing tumors and represent a promising therapy for patients affected by AML.

INV13

Gene therapy for liver diseases — progress and challenges

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Gene therapy (GT) represents a revolution in modern medicine with several products already commercially available and many more in clinical evaluation. Otherwise incurable indications, like most inherited metabolic liver disorders, now have a real therapeutic option. Here we will discuss gene supplementation for the treatment of inherited liver diseases with a particular focus on the use of adeno-associated virus (AAV)-based delivery, with which several clinical trials are currently ongoing with very promising outcomes. Clinical experience has been of foremost importance to highlight key translational challenges associated with this transformative therapy. In particular, the interaction of the patient's immune system with the vector raises some issues about the safety and the duration of treatment efficacy. For instance, several serious adverse events reported in patients after administration of high doses of AAVs suggest a more relevant role of the innate immune responses and pre-existing hepatic conditions than previously thought. We will summarize the main progress, challenges, and potential solutions for gene therapy for liver diseases.

INV14

AAV vectors and the paediatric liver as a therapeutic target

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The liver is a high value target for gene therapy given the plethora of genetic, neoplastic and infectious diseases potentially amenable to genetic and epigenetic intervention. This extends to using the healthy liver as a protein bio-factory for extra-hepatic pathologies. While LNP-mediated delivery of RNA shows immense promise in the liver, recombinant AAV remains the leading system when DNA delivery is necessary and hybrid LNP/AAV approaches are in active development. In the adult liver three AAV-based gene addition therapies have reached registration, all for haemophilia A/B, but to date there are none targeting the paediatric liver. This is largely a consequence of the predominantly episomal nature of AAV and associated loss of vector genomes in concert with liver growth. Beyond the challenge of achieving durable life-long effects, realising maximal therapeutic utility in the paediatric liver also necessitates optimally efficient and safe gene transfer. Increased efficacy is required for robust targeting of diseases with high gene transfer thresholds for therapeutic effect, such as OTC deficiency, while increased safety is vital to reduce the risk-benefit ratios inherent in targeting diseases such as PKU which currently have effective but burdensome therapies. Growing understanding of host-vector interactions observed in pre-clinical and clinical studies, and host-virus interactions in the adult and paediatric population following wildtype AAV infection provide insights and guidance for ongoing vector development. This includes advances in capsid design and pre-clinical evaluation, vector genome architecture and deployment strategies for achieving optimally safe, effective and stable modification of human hepatocytes. Using engineered capsids with progressively improved

human liver tropism, we have explored strategies including transposition of AAV vector expression cassettes into the host cell genome and locus-specific editing by homology-dependent and -independent mechanisms with sequentially improved theoretical safety profiles. Other considerations include reconstitution of physiological gene expression, avoidance of harm to hypomorphic alleles and the logistic utility of mutation agnostic approaches. Finally, we have also explored the use of AAV vectors in combination with LNP-mediated RNA delivery to eliminate the use of promoter-enhancer elements and to improve the fidelity of repair events in the target genome, with the latter remains an ongoing challenge.

INV15

Genetic Vaccine Journey: form cancer treatment to interception

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1: CSO, Nouscom

Viral vectored vaccines are a powerful genetic vaccine platform. Nouscom is developing next-generation, off-the-shelf and personalized viral vector-based cancer vaccines targeting tumor neoantigens. Our approach is based on heterologous prime/boost immunization using non-human Great Ape Adenovirus and MVA vectors. Nouscom lead candidate, NOUS-209, is an off-the-shelf vaccine encoding 209 neoantigens shared across sporadic and hereditary Microsatellite Instable (MSI) tumors developed for interception and treatment of MSI tumors (*Leoni et al., 2020*). MSI tumors are characterized by a defective DNA mismatch repair system, which generates highly immunogenic frame shift peptides (FSPs) that are not found on healthy tissue. Phase 1 study of NOUS-209 vaccine in combination with pembrolizumab (NCT04041310) demonstrated induction ofstrong and durable T cell immune response and clinical benefit in unresectable or metastatic dMMR/MSI-H tumors in 1st and 2nd line settings (*D'Alise et al, STM 2022*). Currently, NOUS-209 is being tested in randomized Phase 2 trial for the treatment of MSI solid tumors in combination with pembrolizumab. One promising approach in the field of cancer immunotherapy is represented by "cancer immune interception", aiming to catch, or intercept, cancer cells as they begin to develop into pre-cancers or very early cancers. Lynch Syndrome (LS) is the most common cause of hereditary colorectal cancer (CRC), with approximately 1 million LS carriers in the US. LS is caused by mutations in one of the four DNA mismatch repair (MMR) genes, leading to tumors that accumulate a large number of neoantigens. The lifetime risk of these individuals of developing CRC is up to a 80%. LS provides an ideal context in which to leverage immune interception strategies, such as NOUS-209 vaccine. Data from a Ph1b/2 trial of NOUS-209 monotherapy in LS carriers will be presented highlighting the potential of NOUS-209 to 'intercept' cancer before it occurs in these individuals

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INV16

Gene therapy for brain tumors using neural stem cells derived from genome-edited iPS cells

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1: Keio University School of Medicine, Tokyo

Malignant glioma is the most aggressive primary brain tumor with a poor prognosis, characterized by diffuse infiltration into the normal brain parenchyma. The development of novel therapeutic modalities is urgently needed. We have shown that human induced pluripotent stem cells (iPSCs)-derived neural stem cells (NSCs) have a high tumor-tropic migratory capacity. Thus, we established the concept of gene therapy using iPSCs-derived NSCs as a cellular delivery vehicle for the treatment of malignant glioma. As a therapeutic gene, we focused on the yeast cytosine deaminase (yCD) suicide gene because of its efficacy and safety demonstrated in clinical trials. In addition, the uracil phosphoribosyl transferase (UPRT) gene was fused to the yCD gene to enhance the anti-tumor effect. To achieve stable transgene expression, yCD-UPRT was inserted into a housekeeping gene locus in iPSCs using CRISPR/Cas9-mediated genome editing. Time-lapse imaging of organotypic brain slice cultures demonstrated the tumor-tropic migratory capacity of iPSCs-yCD-UPRT-derived NSCs and bystander killing of glioma stem cells. Implantation of these NSCs into the brain of human glioma stem cell xenograft mice resulted in a significant therapeutic effect. The suicide gene-expressing iPSCs may be a platform that contributes to the safe regenerative medicine by reducing the tumorigenic risk of stem cell therapy. In this presentation, I will review the gene cell therapy for brain tumors using human iPS cell-derived NPCs and the prospects for future clinical applications.

INV17

Formulation of mRNA sequences in bio-renewable lipid nanoparticles to counter infection with *Mycobacterium tuberculosis* and other pathogens

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Lipid nanoparticles (LNPs) are an important class of synthetic vectors that may be used to deliver therapeutic and antigen-encoding mRNA sequences. They demonstrated excellent properties as carriers of SARS-CoV-2 spike protein-encoding mRNA and were vital to counter the COVID-19 pandemic. Traditionally LNPs comprise four-components, an ionisable lipid, cholesterol, phospholipid and PEG-modified lipid, which package the mRNA. The ionisable lipid is an essential constituent that is required for particle assembly, cellular uptake and endosomal release of mRNA into the cytoplasm. Currently available ionisable lipids are costly, difficult to prepare and derived from products of the petroleum industry. To address these limitations and produce affordable LNPs that may be used for therapy and vaccination, we have developed efficient and inexpensive methods for generating ionisable lipids from bio-renewable sources.

Components of cashew nut shell liquid (CNSL) were used as the primary supply of building blocks for ionisable lipid synthesis. The useful features of CSNL for making ionisable lipids are: 1) unlike other natural oils, CNSL contains three main related lipids, (cardol, cardanol and anacardic acid) which simplifies precursor purification, 2) CNSL is an abundant agricultural waste product that is inexpensive, and 3) the source is bio-renewable.

Hydrogenated cardanol, which naturally contains a 14-carbon unsaturated lipid chain linked to a benzene ring, was purified from CNSL. A panel of ionisable derivatives of this compound was prepared with groups on 1,2,4 or 1,3,5 positions of the benzene ring. Lipid structures were varied by including saturated or unsaturated branched and unbranched side chains of different lengths. Ionisable N atoms were incorporated as amines or within piperazine groups. Further modifications were included to adjust pKas and increase metabolic degradation by including ester groups. Intermediate synthesis steps were high-yielding, and chromatographic purification was only necessary for the final products. These are valuable feature for scalable synthesis required for clinical translation. A panel of approximately 60 ionisable lipids was generated, which were then incorporated into mRNA-delivering LNPs

Four-component mRNA-containing formulations that included the CNSL-derived ionisable lipids were assembled using microfluidics. Encapsulation efficiencies of more than 90% were routinely achieved. Excellent particle diameters, typically approximately 80 nm with polydispersity indices less than 0.3, were consistently obtained. Injection of Firefly luciferase-encoding mRNA showed localised gene expression in vivo following intramuscular and intradermal injection. Some compounds also demonstrated hepatotropism following IM injection, which is a similar feature of ionisable lipids from licensed formulations. LNPs containing mRNAs encoding antigens of SARS-CoV-2 and *Mycobacterium tuberculosis* are being evaluated for suitability as vaccines. Good T cell and humoral immunogenicity has been achieved that compares favourably to licensed formulations. These data lay the foundation for advancing a new class of LNPs with diverse applications. Low-cost and bio-renewable properties are unique features that address some of the limitations of current LNP technology, especially for implementation of vaccination and gene therapy in low- and middle-income countries.

INV18

A multivalent mRNA monkeypox virus vaccine (BNT166) protects mice and macaques from orthopoxvirus disease

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In response to the 2022 outbreak of mpox driven by unprecedented human-to-human monkeypox virus (MPXV) transmission, we designed BNT166, aiming to create a highly immunogenic, safe, accessible, and scalable next-generation vaccine against MPXV and related orthopoxviruses. To address the multiple viral forms and increase the breadth of immune response, two candidate multivalent mRNA vaccines were evaluated pre-clinically: a quadrivalent vaccine (BNT166a; encoding the MPXV antigens A35, B6, M1, H3) and a trivalent vaccine (BNT166c; without H3). Both candidates induced robust T cell responses and IgG antibodies in mice, including neutralizing antibodies to both MPXV and vaccinia virus. In challenge studies, BNT166a and BNT166c provided complete protection from vaccinia, clade I, and clade IIb MPXV. Furthermore, immunization with BNT166a was 100% effective at preventing death and at

suppressing lesions in a lethal clade I MPXV challenge in cynomolgus macaques. These findings support the clinical evaluation of BNT166, now underway (NCT05988203).

INV19

Gene Editing for Glycogen Storage Diseases

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Early treatment is needed to reverse the symptoms of glycogen storage disease (GSD), including GSD Ia and GSD II (Pompe disease). We developed a strategy for genome editing with adeno-associated virus (AAV) vectors vectors using two vectors: a CRISPR vector that expresses Cas9, and a Donor vector that contains a therapeutic transgene. We conducted studies with either Donor vector only (gene therapy) or both Donor and CRISPR vectors (genome editing) to compare these two treatment strategies.

GSD Ia is an inherited metabolic disorder caused by deficiency of glucose-6-phosphatase (G6Pase) that is associated with severe hypoglycemia, glycogen accumulation in the liver and kidneys, and long-term adverse effects including hepatocellular adenoma and carcinoma formation. Gene therapy for GSD Ia is under development, but it fails to stably reverse G6Pase deficiency. We tested two AAV vectors containing: 1) a human *G6PC* transgene (Donor), and 2) a CRISPR vector that targets the *G6pc* gene. Treatment of infant mice with GSD Ia with both Donor plus CRISPR vectors had the highest biochemical correction of liver and highest rates of transgene integration, which correlated with improved survival. After 12 weeks mice receiving genome editing had up to 5.9% transgene integration through homology directed repair. Importantly for safety and efficacy, CRISPR vector genomes were greatly diminished at the end of the study, whereas Donor vector genomes persisted.

Pompe disease is caused by acid α-glucosidase (GAA) deficiency accompanied by lysosomal glycogen in muscle and progressive weakness. We tested two AAV vectors containing: 1) a human *GAA* transgene (Donor), and 2) a CRISPR vector that targets the *Gaa* gene. We administered Donor vector or Donor plus CRISPR vectors to infant *Gaa* knockout mice. Muscle function testing revealed that mice treated with the Donor vector had improved performance, with or without the CRISPR vector. GAA activity was stably increased in the skeletal muscles of Donor and Donor plus CRISPR vector groups to greater than wildtype levels at both 12 and 60 weeks. CRISPR vector treated groups had a very low level of transgene integration. Thus, AAV vectors can directly transduce skeletal muscles and heart, stably correcting GAA deficiency for greater than one year, even in absence of genome editing.

These studies support the following conclusions: 1) Genome editing can integrate a full-length therapeutic transgene in the liver of mice with GSD Ia, and this strategy warrants further development as a more stable treatment for human patients. 2) Transduction of non-dividing striated muscle early in life can stably treat PD, and should be considered for the treatment of infantile-onset Pompe disease.

INV20

Stem Cell Gene Therapy for Childhood Dementias – A Sanfilippo Story

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Therapies to treat the severe neurodegeneration experienced by patients with inherited childhood dementias are lacking. Around 70 percent of lysosomal storage diseases have neurological manifestations, and several of these manifest as childhood dementia, including Sanfilippo diseases (Mucopolysaccharidosis IIIA-D) and the severe forms of Hunter disease (MPSII).

These diseases are caused by defects in genes that are part of a pathway required for the degradation of complex glycosaminoglycans. Defects in one lysosomal enzyme leads to the accumulation of these sugars, and the accumulation of highly sulphated forms of the glycosaminoglycan heparan sulphate in the brain and CSF correlates with the emergence of neurological disease.

The success of using haematopoietic stem cell (HSC) gene therapy to reduce brain symptoms, relies on the genetic modification of blood stem cells using lentiviral vectors to allow supraphysiological enzyme expression. HSC gene modified monocytes traffic to and engraft in the brain as microglial-like cells, delivering increased doses of enzyme directly to the brain.

In this talk, I will describe the preclinical development of an HSC gene therapy for Sanfilippo disease (MPSIIIA) using a CD11b promoter to target myeloid lineages. This human and cell-lineage targeted promoter is likely safer than viral promoters and we have not seen adverse integration events in any of our development programmes.

To leverage the very high peripheral enzyme levels generated by HSC gene therapies, we have further improved this approach to offer a therapy for Hunter disease, by creating a lentiviral vector producing an IDS fusion enzyme using the tandem repeat of the receptor binding domain for ApoE (IDS-ApoEII). Pre-clinical studies have suggested this enzyme will have improved uptake across the blood-brain-barrier.

Thirdly, the wider application of this HSC gene therapy approach has been demonstrated by us using the anti-inflammasome mediator (IL1RA) to correct behaviour and neuropathology in MPSIIIA, as well as further enzyme programmes in MPSIIIB and other lysosomal diseases.

Lastly, I will present data from our phase I/II clinical trial using HSC gene therapy in MPSIIIA (NCT04201405/EudraCT#2019-002051-42), which has treated five children with severe MPSIIIA, aged 6-24 months. All patients are 24-39 months post-HSC gene therapy with no evidence of IMP-related toxicity or safety concerns to date. Rapid and sustained supraphysiological expression of SGSH enzyme in leukocytes, selected lineages and plasma was observed. Cerebrospinal fluid (CSF) SGSH enzyme, previously undetectable, was within or above normal range by six months post-therapy. Abnormal heparan sulfate storage at baseline was rapidly reduced in CSF, plasma and urine, with a normalization of urine glycosaminoglycan ratios. An alteration in the early neurologic phenotype of disease is evident in 4 out of 5 patients, with neurocognitive progression beyond what has been see previously in untreated patients affected by severe MPSIIIA. Longer follow-up is required for all patients to determine the long-term benefit.

Recruitment to our HSC gene therapy trial for Hunter disease (NCT NCT05665166) using IDS.ApoEII is underway, with the first patient enrolled. Together, these data make a compelling case for the development of further HSC gene therapies for childhood dementias.

INV23

Genetic reprogramming of tumor-associated myeloid cells to awaken anti-cancer immunity

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The successful application of adoptive T-cell therapies in patients with solid tumours presents several challenges, in particular the paucity of tumour-specific targets, intratumour heterogeneity and the presence of a hostile tumour microenvironment. Myeloid cells co-opted by the tumour are largely responsible for an immunosuppressive microenvironment that often hinders T-cell mediated tumour rejection. I will present strategies by which myeloid cells can be genetically reprogrammed from protumoural to antitumoural actors. To ensure a constant and durable anti-tumour effect, the strategy is based on the transplantation of genetically modified haematopoietic stem and progenitor cells that continuously produce large amounts of tumour-infiltrating myeloid cell progeny, which act as a Trojan horse to replenish the tumour macrophage pool as the tumour progresses. As an example, the journey from bench-to-bedside of an HSPC product that specifically delivers interferon into the microenvironment of tumors will be presented, starting from preclinical development to the preliminary results of a phase I/IIa dose finding study in patients affected by glioblastoma multiforme (NCT03866109). An outlook will be given on how this concept could be the subject of refinement and expansion in the future.

INV24

Overcoming oncolytic virus resistance to improve the treatment of pediatric brain tumors

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Pediatric high-risk brain tumors remain the leading cause of cancer-related death in children. For the last 30 years, all treatment approaches for the most aggressive types of these tumors have failed, leaving a terrible prospect of survival at five years for these children virtually zero. Thus, it is clear that new therapeutic strategies are required to allow for more effective treatments of these tumors and defer the severe side effects derived from the current therapeutic choices. Oncolytic adenoviruses designed to replicate in and destroy tumor cells selectively represent a promising therapeutic strategy that could improve the outcome of this malignancy. DNX-2401 (tasadenoturev; Delta-24-RGD) is a tumor-selective oncolytic virus evaluated in phase 1 and 2 clinical trials in adults with glioblastoma with promising results. These studies provide evidence for monotherapy activity after a single DNX-2401 dose, including complete responses and immune responses against the tumor. Taking advantage of the experience with DNX-2401 in adult brain tumors, we have dedicated our efforts to characterize the preclinical and clinical

efficacy in pediatric brain tumors. Preclinical studies demonstrate anti-tumor effects, allowing us to open the first-in-human clinical trial for pediatric patients with diffuse intrinsic pontine glioma (DIPG). Twelve patients were treated with DNX-2401 and standard-of-care radiation. The procedure was well-tolerated, and clinical responses and survival benefit was observed. The immune cell composition of the biopsies pre-virus injection was assessed using multiplexed quantitative immunofluorescence. T cells were hardly noticeable in these tumors, while macrophages were abundant. Preclinical and clinical data highlight the importance of the immunotherapeutic arm of Delta-24-RGD in mediating durable anti-tumor responses. In this matter, Delta-24-RGD derivatives encoding immunostimulatory transgenes have been developed to enhance the immunotherapeutic component of the treatment. Lessons learned from the clinic allowed us to engineer other viruses based on the Delta-24 platform to boost the immune response. Additionally, combining immunomodulatory approaches or small molecules already used in the clinic with the oncolytic virus could constitute a faster approach to implementing their translation to the clinical setting and overcoming regulatory issues. In this work we will discuss several approaches used to bypass resistance and increase efficacy of oncolytic viruses for the treatment of pediatric brain tumors.

INV25

Programming permanent gene repression by epigenetic editing

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Epigenome editing is emerging as a powerful new strategy to silence gene expression without altering the primary DNA sequence. In this context, we and others have previously demonstrated that transient delivery of Engineered Transcriptional Repressors (ETRs) can result in efficient, long-term, stable, and specific epigenetic silencing of endogenous genes in both human and mouse cells, and more recently, in vivo in mice. ETRs are chimeric proteins consisting of a programmable DNA-binding domain, such as CRISPR-Cas9 or ZFPs, fused to one or more effector domains derived from naturally occurring epigenetic repressors. During my talk, I will present our efforts to refine and characterize this technology, focusing on its in vivo and ex vivo applications for the treatment of monogenic and acquired diseases.

INV26

Unlocking the unreachable: harnessing tRNA therapeutics for rare genetic conditions

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11% of all genetic mutations diseases are linked to non-sense mutations that convert a sense codon decoded by tRNA into a premature termination or stop codon (PTC), resulting in an abrupt termination of translation. To correct PTCs, we developed a novel strategy which is based on

repurposing sense-codon decoding tRNAs to readthrough nonsense mutation-associated premature termination and restore protein synthesis and function. I will discuss our recent advances in the development of tRNA therapeutics with high activity and safety in cell and animal models and discuss different formulation approaches for single or chronic treatment modalities.

INV27

Hematopoietic stem cell gene therapy: Overcoming challenges in Fanconi anemia

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Gene therapy for Fanconi anemia (FA) has been a major challenge for many years since none of the previous trials had been able to demonstrate engraftment of gene corrected hematopoietic stem cells (HSCs) in treated patients. Our laboratory has focused on bringing this therapeutic alternative to FA patients in a phase I/II clinical trial (FANCOLEN-1). Mobilized peripheral blood CD34+ cells from nine FA-A patients were transduced with a therapeutic *FANCA*-encoding lentiviral vector and reinfused without any preconditioning treatment. Long-term follow-up in this clinical trial has shown a progressive increase in genetically and phenotypically corrected lympho-hematopoietic cells in 6 out of 8 evaluable patients. Importantly, 2 of the 3 patients who were infused with at least 240,000 corrected CD34+ cells/kg showed correction of the BMF progression. These results paved the way for the development of a global phase II trial sponsored by Rocket Pharmaceuticals, in which higher numbers of corrected CD34+/cells were infused at an early stage of the disease. As a result, faster engraftment and progressive increases of corrected BM and PB cells have been observed so far in 8 of 12 evaluable patients over 15-54 months of follow-up.

The results of the LV-mediated gene therapy trials and the development of novel genome editing tools, such as prime editing (PE), prompted us to test the feasibility of this novel editing approach to correct specific mutations in HSCs from FA patients. The PE3 system demonstrated high performance in healthy donor HSPCs both in vitro and in vivo. More recently, purified BM CD34+ cells from FA patients carrying the FANCA c.295C>T mutation were edited by PE. Current data has confirmed that PE efficiently reverts the mitomycin C hypersensitivity of FA HSPCs.

Our results highlight the relevance of LV-mediated gene therapy as a new alternative to correct BMF in FA patients without genotoxic conditioning, and demonstrate the potential of prime editing as a future alternative for specific mutations in different FA genes.

INV28

Discovery and development of CRISPR-associated transposases for RNA-guided gene insertion

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In recent years, genome editing technologies have advanced from nuclease-based methods that generate DNA double-strand breaks (DSBs), which can cause undesired byproducts, to next-generation CRISPR approaches that perform controlled chemistry using DSB-independent strategies. Base editing and prime editing are ideally suited for small-scale modifications, but methods to achieve large-payload gene insertion have been lacking. To address this gap, I will present work from my lab describing a new family of CRISPR-associated transposases (CAST) that perform highly accurate, targeted integration of DNA payloads via RNA-guided transposition. Unlike conventional CRISPR systems that combine targeting and cleavage, CAST systems exploit nuclease-deficient CRISPR systems for RNA-guided DNA targeting, leading to the site-specific recruitment of transposase enzymes for DNA insertion. We have resolved molecular details of this pathway using a combination of high-throughput sequencing, biochemistry, genetics, and cryo-electron microscopy, revealing a hierarchical assembly pathway and a structural roadmap to guide engineering efforts. More recently, we leveraged phage-assisted continuous evolution (PACE) to identify transposase variants with ~200-fold improved activity in mammalian cells, yielding 10–25% integration efficiencies of kilobase-size DNA cargos across several human genomic sites. Our long-term goal is to harness CAST as a versatile platform technology for integrating therapeutically relevant DNA payloads into the genome to treat human disease.

INV29

Capsid engineering to empower the adeno-associated virus (AAV) vector system for its new "fields of action"

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AAV vectors, biological nanoparticles derived from replication-deficient adeno-associated viruses (AAV), are currently the most widely used delivery system for *in vivo* gene therapy. One of the best indicators of the successful translation is the increasing numbers of market-approved AAV vector-based *in vivo* gene therapies that target distinct monogenetic diseases, which are entering clinical routine. The decision of whether to apply AAV vectors locally or intravenously is dependent upon the disease and, thus, dependent upon the main target cell type. Compared to *ex vivo* gene therapy strategies, *in vivo* applications face the challenge of the high prevalence of pre-existing neutralizing antibodies in the human population due to exposure to the wild-type virus. Moreover, even when applied locally, vectors will be lost in off-target cell types due to the generally broad tropism of vectors based on naturally occurring viruses. A further challenge is the high vector dose that is applied to overcome pre- and post-entry barriers towards transduction. In response to these challenges, many efforts are invested in capsid engineering, a technique that entered the field of AAV vector development more then 25 years ago. Since then, the AAV

toolbox has been greatly expanded with first capsid engineered AAV vectors in human clinical trials. Equipped with a portfolio of capsid engineering strategies and a continuously increasing knowledge of the host-vector interactions that impact the efficacy of AAV vector-mediated cell modification, the potential medical utility of AAV vectors is expanding to new “fields of action”. For example, capsid engineered AAV vectors are explored as vaccine platforms, to improve outcome for patients receiving implants, to improve *ex vivo* gene therapy approaches and to move *ex vivo* gene therapies into *in vivo* applications.

INV30

Personalized preclinical gene editing protocols for *ex vivo* and *in vivo* correction of rare skin disease

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We propose preclinical strategies in experimental models (humanized skin) for two of the most severe genodermatoses, recessive dystrophic epidermolysis bullosa (RDEB) and pachyonychia congenita (PC). RDEB is a skin fragility disorder characterized by recurrent lesions with impaired healing caused by loss-of-function mutations in COL7A1, the gene encoding collagen VII (C7), whereas PC is a keratinization disorder caused by dominant mutations in *the genes encoding keratins K6, K16 or K17*. We propose to generate tools for precise correction in patient cells through permanent correction by reframing in the case of EBDR patients and inactivation of the mutant allele in the case of PC. In both models, we will develop and test CRISPR RNPs-based therapies as first ex vivo approaches and adenoviral vectors to advance in vivo therapies by NHEJ gene editing of *COL7A1* and *KRT6A*.

INV31

In situ gene editing as a topically applicable treatment option for genodermatoses

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Gene therapies are powerful tools to prevent, treat, and cure human diseases. The application of gene therapies for skin diseases received little attention so far, despite the easy accessibility of skin and the urgent medical need especially in the context of genodermatoses. A major obstacle is the unique barrier properties of human skin, which significantly limits the absorption of biomacromolecules, and thus hampers the efficient delivery of nucleic acid payloads.

In this presentation, I will discuss our recent work on developing a topically applicable CRISPR-based gene therapy as novel treatment option for genodermatoses with a focus on congenital ichthyosis. I will present our efforts to identify skin-optimized lipid nanoparticles as lead formulations, our approach of correction, its platform potential, promising preclinical data on *in situ* correction efficacy and functional restoration as well as initial *in vivo* toxicity data.

INV34

Highly efficient in vivo editing of neurons via peptide-mediated delivery of CRISPR enzymes

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CRISPR-driven genome editing within the central nervous system (CNS) has the potential to revolutionize the treatment of neurological disorders, including monogenic neurodegenerative diseases such as Huntington's, Alzheimer's, and Parkinson's diseases. However, developing CRISPR therapeutics for the CNS has been challenging, due in large part to delivery. Two key obstacles need to be overcome before gene editing in the human brain is feasible: (1) efficient and safe delivery of editing machinery into neurons, and (2) establishment of strategies that enable targeted brain regions to be efficiently edited.

Our team has advanced an approach for highly efficient editing of neurons in the brain. This approach relies on the intersection of three technologies: NLS-rich CRISPR ribonucleoprotein (RNP) enzymes that possess some capacity for self-delivery into cells, amphiphilic peptides that substantially enhance efficiency of intracellular RNP delivery, and convection-enhanced delivery (CED), a means of administration that promotes optimal distribution after local injection. This approach results in widespread, high-efficiency genome editing of neurons in the mouse brain following administration into striatum, thalamus, or hippocampus. This approach is being used to edit neurons in the brain of a large mammal (pig) and is being evaluated for its capacity to provide phenotypic correction in a murine model of Huntington's disease.

Preliminary evaluation demonstrates favorable host responses: negligible toxicity and only moderate immune responses to the reagents. Our approach relies on only three components – CRISPR protein, guide RNA, and synthetic peptide – all of which are straightforward to manufacture at scale. The transient nature of our reagents provides the benefits associated with non-viral delivery: limited risks of off-target genome editing or immune responses to the CRISPR cargo. In contrast to lipid nanoparticle (LNP) delivery of mRNA cargo, which are typically at least 100 nm in diameter, our particles are an exceptionally small ~20 nm in diameter, permitting diffusion through the brain parenchyma, which has a pore size of ~50 nm. Together, these properties suggest our approach for CRISPR enzyme delivery has substantial potential for clinical translation in addressing neurological diseases.

INV39

Innovations in Factor VIII Biology for Next Generation Gene Therapy for Hemophilia A

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The development of therapeutics for hemophilia A has benefited from insights from studies of factor VIII (FVIII) biology. The FVIII variant, B-domain deleted human FVIII (hFVIII-BDD), has been used in protein therapeutics and gene therapy for several decades. FVIII variants that have improved secretion, higher specific activity or are inactivation resistant have been characterized and introduced into gene therapy approaches. Based on our studies of canine FVIII, we identified a unique hFVIII-BDD protein variant, hFVIII-Δ3-SP/DE, that has a minimal modification of its proteolytic cleavage sites to make it more similar to the canine protein at these sites. In vitro studies show that this variant has higher biological activity and is secreted more efficiently. Introduction of hFVIII- Δ3-SP/DE into adeno-associated viral (AAV) vector constructs results in higher levels of FVIII expression compared to hFVIII-BDD in hemophilia A mice, hemophilia A dogs and non-human primates. This FVIII variant offers a novel strategy for improving the efficacy of AAV gene therapy and reducing the AAV vector dose while overcoming a key barrier in AAV-mediated gene therapy for hemophilia A. The use of gain-of-function FVIII variants provides an opportunity to overcome the limitations of AAV-based gene therapy for next generation gene therapy approaches for hemophilia A.

INV40

IPEX Syndrome Phase 1 gene therapy with engineered CD4^{lvfoxp3} Treg-like cells

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A First-Time-in-Human (FTiH) Phase 1 clinical trial with a novel regulatory T (Treg) cell therapy product to treat IPEX syndrome is ongoing (NCT05241444). IPEX is a life-threatening monogenic autoimmune disease caused by loss-of-function FOXP3 mutations and dysfunctional Treg cells.

We administer autologous engineered $CD4^{LVFOXP3}$ Treg-like cells, lentiviral vector (LV)-transduced CD4+ T cells expressing wild-type human *FOXP3* cDNA resulting in the acquisition of Treg-cell phenotype and function.

Primary objectives are feasibility of manufacturing $CD4^{LVFOXP3}$ cells, safety of maximum tolerated dose of $CD4^{LVFOXP3}$ in two different age cohorts. Secondary objective is to assess the impact of $CD4^{LVFOXP3}$ infusion on clinical manifestations. We are testing three escalating doses in a 2+4 trial design.

A total of six patients have been treated. Of the first four IPEX patients treated in > 12-year-old cohort two received the lowest dose ($1x10^6$/kg) and two the intermediate dose ($3x10^6$/kg). In addition, two patients <12 yrs of age received the intermediate dose. We have demonstrated the feasibility of GMP manufacturing at high-purity $CD4^{LVFOXP3}$ cells and the safety of the infusion. $CD4^{LVFOXP3}$ expresses membrane tNGFR (CD271) from the same LV construct that expresses the FOXP3 cDNA, allowing ex vivo purification during manufacturing and in vivo traceability. Pharmacokinetics (PK) show that $CD4^{LVFOXP3}$ are detectable in blood and tissue, and their phenotypic and functional stability is preliminary confirmed at the single cell level. Combining single-cell CITE and TCR sequencing, and other methods to investigate the CD4+T cells from IPEX patients before and after manufacturing of $CD4^{LV\text{-}FOXP3}$, we found that $CD4^{LV\text{-}FOXP3}$ subsets have higher expression of the transgene *FOXP3-lv*, memory marker gene *SELL*, *CD27*, Treg marker genes, including *CCR4*, *CTLA4* and *IL2RA*, lower expression of *IL7R* and the inflammatory cytokines *IL17A*, *IL17F*, *IL22*, *IL15*, *IFNG*, constrained TCR clone expansion, and cycling status. More intriguing, we found that (1) the most dominant subset of $CD4^{LV\text{-}FOXP3}$ cells have the stem cells memory features, which are most likely derived from naïve CD4+ T cells. (2) $CD4^{LV\text{-}FOXP3}$ cells can survive one year and maintain the expression of the transgene *FOXP3-lv* over time we monitored. Thus, these findings reveal that $CD4^{LV\text{-}FOXP3}$ products preserve their Treg-like signatures, maintain the stemness from naïve cells, and keep their identity in severe inflammatory environment in IPEX patients.

Successful completion of this trial will address a significant unmet medical need while also providing proof of safety and preliminary indications of benefit in controlling autoimmune disease manifestations. As such, these data could lead to expanded application of autologous $CD4^{LVFOXP3}$ to other more common autoimmune disorders.

INV41

Accelerating genome-based therapies for neurological diseases

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Oligonucleotide therapies have potential for treating a wide range of inherited and acquired disorders through modulating gene expression e.g. via splice modulation, gene silencing or novel mechanisms. Inherited monogenic disorders such as Duchenne muscular dystrophy (DMD), are classical examples where modulation of pre-mRNA splicing of the *DMD* gene can restore viable reading expression of functional protein to achieve disease modification. While significant progress has been made with FDA approval of the numerous oligonucleotide therapeutics for disorders such as DMD and spinal muscular atrophy (SMA), a major challenge is overcoming poor

oligonucleotide delivery to extra-hepatic tissues to enable more effective and wider application of oligonucleotide therapeutics. A second challenge is to increase the speed of therapeutic oligonucleotide development to address unmet clinical need across neurological and neuromuscular diseases. Current progress and future prospects will be discussed.

INV42

Cell Specific Delivery of RNAs Using Targeted Lipid Nanoparticles: from Vaccines to Therapeutic Genome Editing

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Objectives: Modulating gene function that can lead to cell death in the case of tumors or to change of expression of desired proteins for vaccines or editing of cells for knockout or knockin of genes is a major challenge and an unmet need.

Methods: We have developed several strategies to target unique cell surface receptors and as well as a very large library of ionizible lipids with two special features: highly immunogenic or less immunogenic (that can be used for repeated doses). In addition, we developed novel mRNA sequences with more potent immunogenicity that can be utilized against bacterial infection.

Results: In this talk, I will share several examples ranging from new therapeutic targets identified for cancer (e.g. CKAP5) to the first bacterial mRNA vaccines and will also share some data on gene editing for cancer therapeutics.

Conclusions: Cell specific delivery of RNA payloads is feasible and represent the next generation milestone in personalized medicine.

INV46

Switching genes on and off during haematopoiesis

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We study how transcriptional and epigenetic programmes are played out on chromatin spanning the alpha globin cluster as hematopoietic cells undergo lineage fate decisions and differentiation to form erythroid cells. The alpha globin cluster and its regulatory elements are silenced in early

progenitors, poised for expression in later progenitors and fully expressed during terminal erythroid differentiation. Using a variety of approaches we have established the order in which silencing factors are removed, activating transcription factors bind and epigenetic modifications occur. In addition, we have shown how chromosomal conformation and nuclear sub-localisation change during hematopoiesis. Natural cis and trans acting mutations that cause alpha thalassaemia provide additional insight into how the long-range regulatory elements may interact with the promoters of the globin genes and other flanking genes to activate their expression. Together these observations establish some of the general principles by which genes within their natural chromosomal environment are switched on and off during differentiation and development and how these processes are perturbed in human disease.

INV47

T-cell engineering to fight cancer and autoimmunity

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The potency of our immune system in restoring and maintaining homeostasis and health is well known. In particular, T lymphocytes, major actors of the adaptive immunity, are critical players in several diseases, including infections, cancer and autoimmunity. This notion coupled with the most recent gene transfer and genome editing technologies have prompted the development and use of adoptive cellular therapy with engineered lymphocytes as an emerging therapeutic modality. For cancer treatment, this therapeutic approach stems from the pioneering work, based on naturally occurring T cells, that demonstrated the ability of T cells to mediate anti-tumor clinical responses. In the autologous setting, expanded tumor-infiltrating lymphocytes obtained from resected solid tumors, such as metastatic melanoma, proved capable of recognizing tumor-associated antigens and to induce complete remissions once reinfused to patients. In the allogeneic setting, donor lymphocyte infusions after allogeneic hematopoietic stem cell transplantation, has been used to exploit the alloreactive graft versus leukemia effect of donor lymphocytes, although at the price of a major complication, named graft versus host disease.

Gene transfer and genome editing allow today to genetically engineer T cells to increase their fitness and persistence, to improve their safety profile, to redirect their specificity, and to endow the cellular products with new functions, such as the ability to resist the tumor microenvironment. By enabling the transgenic creation of antigen-specific T cells, genetic engineering technologies have widely broadened the use of adoptive T cell therapy. In recent years, the U.S. Food and Drug Administration and European Medicines Agency have approved six engineered T-cell therapeutic products, all chimeric antigen receptor (CAR) engineered T cells directed against B cell malignancies. In parallel, T-cell receptor (TCR) gene therapy is emerging as a promising approach for the treatment of several additional tumors.

Increasing knowledge in immunology, gene transfer and genome editing is opening a new window of opportunity for this new class of therapeutics. While cancer is the major clinical indications for engineered T cells, autoimmunity represents a new frontier, already crossed with success with the first therapeutic products. Although several hurdles, including manufacturing complexity and costs still limit their wide use, these living drugs, able to persist for decades after infusion, represent a completely new and successful therapeutic paradigm for several severe diseases.

INV48

In vivo genome editing in mice and macaques using mRNA-LNP delivery

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The use of mRNA lipid nanoparticles (mRNA-LNP) for the delivery of genome editors into the liver has emerged as a powerful alternative to traditional viral delivery methods. This approach offers several key advantages, including a safer profile with no risk of insertional mutagenesis, a transient duration of gene editor expression, the ability to deliver larger genetic payloads, and the potential for repeat dosing. Recent advancements have leveraged mRNA-LNP in both preclinical and clinical settings to deliver a range of gene-editing tools such as CRISPR-Cas9 nucleases, zinc-finger nucleases, TALENs, and base editors.

In my presentation, I will discuss our latest progress in using mRNA-LNP for base- and prime-editing applications in the livers of mice and macaques. Our work demonstrates effective in vivo correction of mutations associated with Phenylketonuria (PKU), a metabolic liver disorder characterized by elevated phenylalanine levels that result in neurotoxicity. Additionally, we have conducted a detailed spatial analysis of editing rates within liver lobules by adapting imaging-based in situ sequencing techniques. Our analysis reveals similar editing rates after mRNA-LNP delivery in periportal and pericentral hepatocytes, which are functionally distinct and involved in different genetic liver diseases.

Overall, our findings underscore the potential of mRNA-LNP mediated genome editing as a versatile therapeutic tool for the treatment of a wide range of genetic liver diseases.

INV49

Impact of Gene Editing on HSPC Dynamics in Macaques

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Many applications in hematopoietic stem cell gene therapy require approaches that correct or completely replace genetic sequences. In addition, emerging data demonstrates that gene addition approaches relying on semi-random vector insertion, even with less genotoxic lentiviral vectors, have some risk of genotoxicity. We have focused on utilizing the rhesus macaque autologous transplantation model as the most predictive of outcomes in humans, based on phylogenetic, lifespan, size and HSC physiologic similarities. Based on concerns regarding the impact of various precision editing approaches on HSCs, we utilized quantitative genetic barcoding to track the long-term impact of these technologies on HSC engraftment and dynamics. To track the output and clonality of HDR-edited cells and to provide a comparison to lentivirally-transduced HSCs, we utilized a competitive macaque autologous transplantation model, co-infusing HSCs transduced with a barcoded GFP-expressing lentiviral vector (LV) and HDR edited at the CD33 locus. CRISPR/HDR-edited cells showed a two-log decrease by 2 months following transplantation, with little improvement via inhibition of p53 pathways, in comparison to minimal

loss of LV-transduced cells long term. HDR long-term clonality was oligoclonal in contrast to highly polyclonal LV-transduced HSCs. We applied similar approaches to study the impact of ABE base-editing, also documenting substantial but less profound impact on HSCs. These results suggest marked clinically relevant differences in the impact of current genetic modification approaches on HSCs.

INV50

Dissecting Cell Intrinsic Innate Immunity against Viral Vectors

A Kajaste Rudnitski [1]
1: University of Pavia

The low gene manipulation efficiency of human hematopoietic stem cells (HSC) and other clinically relevant target tissues remains a major hurdle for sustainable and broad clinical application of innovative therapies for a wide range of disorders. Current and emerging gene transfer and editing technologies expose cells to components potentially recognized by host antiviral factors and nucleic acid sensors that likely restrict their genetic engineering and contribute to broad individual variability in clinical outcomes. In this context, our research is focused on identifying the antiviral factors and innate sensing pathways that prevent efficient modification of HSC and other target tissues relevant for gene therapy such as cells of the hematopoietic and central nervous systems with the aim of mitigating their effects using methods developed through the understanding of their mechanisms of action. Thus far, our studies on vector-host interactions have uncovered substantial differences in how HSC sense distinct viral vectors and identified potent innate immune blocks to gene transfer and editing that are constitutively active in HSC, leading to the development of novel, highly efficient gene therapy approaches. In this talk, I will review and illustrate recent advances in our understanding of how pathogen recognition and consequent innate immune signaling may affect the efficacy and safety of gene therapy approaches across delivery platforms and clinically relevant target tissues.

INV51

Lipid nanoparticles do not contain intrinsic adjuvants activity

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LNPs recently emerged as a safe and powerful tool to deliver mRNA vaccines *in vivo*. They consist of four different types of lipids: cholesterol, PEGylated lipids, phospholipids and an ionizable lipid. The ionizable lipid has been proposed to facilitate exit of the antigen from the phagosome into the cytosol, thereby promoting antigenicity. The recent success of mRNA-LNPs pushed the LNP technology to the forefront of medicine. Still, many questions remain, and there is an urgent need

to understand the molecular principles behind their mode of action. One of the most remarkable properties of mRNA-LNPs is that they do not require additional adjuvants to induce potent immune responses, in contrast to most classical vaccines. Whether the lipid components of the LNPs can serve as a stand-alone driver of adjuvanticity of mRNA-based vaccines without the need for mRNA components remains a conundrum, though. Our previous data revealed that empty LNPs (eLNPs) – in contrast to polyinosinic:polycytidylic (pIC) acid-adjuvanted LNPs (pIC-LNPs) - induce homeostatic and not immunogenic dendritic cell (DC) maturation *in vivo* (Bosteels et al., Sci Imm, 2023), suggesting that the LNP itself may not be sufficient to induce a potent T cell immune response.

As this contrasts with other reports, we decided to apply an unbiased approach to dissect how different types of LNPs (adjuvanted with Toll-like receptor (TLR) ligands or non-adjuvanted) steer DC maturation pathways *in vivo* and performed a CITE-SEQ experiment on splenic DCs isolated at different time points after LNP-injection. Both the expression of homeostatic/immunogenic cell surface markers and the transcriptional profile induced unequivocally demonstrate that empty, non-cargo containing LNPs are not able to induce immunogenic DC activation, in contrast to mRNA-LNP or TLR-adjuvanted LNPs. This is different from what has been observed for other cell types where uptake of eLNPs does induce an inflammatory response. It reveals that DCs are well equipped to decode adjuvanticity at a very sensitive scale and that the mRNA cargo has an essential contribution in the adjuvants activity of mRNA-LNPs. Our data also indicate that the scope of therapeutic applications of LNPs might be broader than originally anticipated and that they may have potential as vehicle to induce tolerance against non-immunogenic cargo.

INV52

Activity, biological properties, and safety of non-viral CAR T cells engineered with Sleeping Beauty for hematological malignancies

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CAR T-cell immunotherapy has revolutionized the treatment landscape for B-cell neoplasms, with six CAR T-cell products currently approved worldwide. However, challenges remain, including high costs, complex manufacturing, and the risk of toxicities due to immune activation. Non-viral engineering, such as the Sleeping Beauty transposon system, is a promising option to offer a more streamlined and cost-effective approach while still achieving stable gene transfer. SB-engineered CAR T cells was successfully tested in clinical trials, with 45 patients treated for B-ALL.

Here, we report the results from 36 patients (4 children and 32 adults) treated with CARCIK-CD19 in a pooled analysis of Phase 1/2, compassionate base use, and Phase 2 trials, sponsored by Tettamanti Center, Fondazione IRCCS San Gerardo dei Tintori, Monza. The trials demonstrated encouraging safety and efficacy, with a high remission rate and improved overall survival, associated with expansion and persistence of SB-engineered T cells. Notably, Integration site analysis after infusion showed no integration bias toward gene-dense regions, reducing the risk of insertional oncogenesis.

Transcriptional analysis of CAR T cells and the BM microenvironment from treated patients was performed and findings were validated by flow cytometry. We observed profound changes in the

composition of BM after CAR T-cell infusion compared to pre-treatment samples, with an increase in the myeloid cells, and exhausted CD8+ T cells. Interestingly, myeloid cells displayed a higher resemblance to myeloid-derived suppressor cells (MDSCs) post-infusion, with GeneSet Enrichment Analysis highlighting immunosuppressive pathways. These pathways are closely related to the development of terminally exhausted T cells and MDSCs, suggesting that CAR T-cell-mediated myeloid activation may contribute to immune dysregulation, limiting CAR T-cell expansion and potentially reducing therapeutic efficacy.

Efforts are underway to extend CAR T-cell therapies to acute myeloid leukemia (AML). The SB platform is being explored for targeting leukemia-initiating cells (LICs) in AML, using CD117 as a target antigen. This approach aims to eradicate AML cells while also serving as a conditioning regimen for subsequent hematopoietic stem cell transplantation (HSCT), without the need for genotoxic conditioning. To allow for HSCT rescue while preventing permanent myeloablation, antigen-versatile targeting and effector off-switch mechanisms have been achieved by adaptor-CAR (AdFITC-CAR) T-cells in combination with AML antigen-binding diabodies. In therapeutic xenogeneic mouse models, AdFITC-CAR T-cells co-administered with fluoresceinated diabody adaptors were as efficient as direct CAR T-cells, and combinatorial use of adaptors further enhanced therapeutic efficacy.

In summary, the non-viral SB-engineered CAR T-cell platform shows great potential for improving the safety, cost, and efficacy of CAR T-cell therapies across hematologic malignancies.

INV53

Overcoming current limitations in CAR T-cell therapy of ALL

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CD19-directed CAR T-cell therapy for precursor B-cell ALL represents an exemplar of the curative potential of this immune therapy and was the first licensed indication for CAR T-cells. However, less than half of patients treated are cured. I will discuss the limitations of CAR T-cell therapies in this context, the steps taken to improve outcomes and their impact to date.

INV57

The Economics of Gene Therapies

DM Epstein [1]
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As of 2023, 19 advanced therapy medical products (ATMP) had obtained marketing authorization in the European Union, but only 13 had translated successfully into clinical practice. Moreover, access to ATMPs is uneven across European countries. Successful development of gene therapies, and access by patients to them, depends on alignment of interests and decision making

across multiple stakeholders. This presentation focuses on the challenges facing three broad categories of stakeholders: developers, regulators, and health service payers.

At the research and development stages, the importance of synergistic research groups and public- private collaboration is crucial. Discoveries in fields outside the life sciences, such as artificial intelligence and gene editing, are increasingly important in facilitating R&D in gene therapy. The interplay between public and private R&D is complex. Many scientists move between academia and industry during their careers, bringing with them their knowledge, skills and sometimes their intellectual property. A balance needs to be struck between providing the right individual and corporate incentives to encourage R&D, while ensuring that the knowledge is widely disseminated and can be exploited by others. The role of specialist investor capital is crucial especially during the "translational gap" in preclinical development.

The regulatory framework for gene therapies is complex. There are differences between European countries in how they regulate hospital exemption and this can be one source of variation in access. Regulators offer a number of incentives to encourage products that address unmet need or rare diseases, including adaptive pathways for market access and extended periods of exclusivity and data protection, that often apply to gene therapies. Nevertheless, some commentators warn that incentives for orphan drugs should not become so generous to the extent of distorting R&D effort away from other areas of greater public health benefit.

Health Technology Assessment (HTA) is the process by which health service payers evaluate comparative effectiveness, value for money and other attributes of innovations, in preparation for pricing and reimbursement decisions. The forthcoming EU regulation of HTA may avoid duplication and align criteria for clinical domains to some extent, but will not address challenges arising from deficiencies in the evidence base, uncertainty about long term impacts and quality of life, or high prices. Innovative payment schemes, such as outcome-based reimbursement, may address some of the risks and uncertainties but should not be considered a substitute for a rigorous HTA process. HTA would be facilitated by greater interoperability and sharing of data, alignment between evidence requirements of medicine regulators and HTA bodies, more transparency from all stakeholders, and clarity about how pricing and reimbursement decisions are made.

INV64

Engineering B cells for custom antibody production

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We reprogram B cells to express custom antibodies and antibody-like molecules through homology-directed genome editing at the immunoglobulin heavy chain locus (IgH). This allows us to express antibodies that are not naturally induced by vaccination, such as anti-self antibodies, or broadly neutralizing anti-HIV antibodies that control HIV *in vivo*. The editing protocol inserts custom antigen-recognizing domains (antibody 'heads') into constant regions of IgH, resulting in molecules that mimic the heavy chain only antibodies (HCAbs) made by Camelids. Incorporation of Fc mutations is also possible, enhancing antibody effector functions and preventing interactions with ay endogenous antibody chains.

We hypothesized that this approach would preserve important features of natural antibody expression, including the response to antigens and subsequent differentiation towards antibody-secreting plasma cells and memory cells. To assess the function of IgH-edited B cells we

engineered both human and macaque B cells with a panel of anti-HIV molecules. We confirmed the function of the human B cells using a tonsil organoid model that recapitulates the vaccination response. For macaques, we transplanted animals with autologous B cells engineered to express either of two alternate anti-HIV domains and evaluated HCAb levels in blood over time. Our goal is engineer B cells to express anti-HIV molecules with enhanced breadth and potency that respond to HIV reactivation of vaccination after transplantation. In this way we envision a long-lasting cell therapy that could control HIV and dynamically respond to viral loads

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INV68

Beyond capsid-modifications: Development of genome-modified AAV vectors

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1: University of Florida

A number of investigators, including us, have developed capsid-modified next generation (NextGen) of AAV vectors for high-efficiency transduction. However, we reasoned that beyond capsid-modifications, it is equally important to pursue genome-modifications to improve transgene expression from AAV vectors since it is the genome, more specifically the viral inverted terminal repeats (ITRs), beyond the promoters an enhancers, and not the capsid, that plays an important role in modulating the expression of the therapeutic transgene. We have previously reported the development of genome-modified generation X (GenX) AAV vectors in which deletion of one of the D-sequences in the AAV-ITRs mediated up to ~8-fold higher transgene expression from single-stranded AAV (ssAAV) vectors in human cells *in vitro* and in mouse hepatocytes *in vivo*. We recently reported the development of genome-modified generation Y (GenY) AAV vectors in which the replacement of the distal 10-nucleotides in the D-sequence with the glucocorticoid receptor-binding element (GRE) led to up to ~10-fold higher transgene expression from ssAAV vectors in human cells *in vitro* and in murine hepatocytes *in vivo*. More recently, we also reported the development of generation Z (GenZ) ssAAV vectors in which replacement of the proximal 10 nucleotides in the D-sequence with a random N10-sequence from a library resulted in ~20-fold increased transgene expression from these vectors in human cells *in vitro* and in mouse hepatocytes *in vivo*. We have also combined the features of GenY and GenZ ITRs to develop a novel AAV vector, designated DeltA AAV vector. DeltA AAV vectors mediate up ~30-fold higher levels of transgene expression in differentiated primary human skeletal muscle cells *in vitro* and in murine skeletal muscles *in vivo* compared with that from the conventional ssAAV vectors. Thus, beyond capsid-modifications, it is equally important, perhaps more so, to pursue ITR-modifications to improve transgene expression from ssAAV vectors. Encapsidation of these ITR-modified GenX, GenY, GenZ, and DeltA genomes into capsid-modified NextGen capsids, resulting in optimized (Opt^{X}, Opt^{Y}, Opt^{Z}, and Opt^{DeltA}) AAV serotype vectors is likely to significantly improve the performance of these vectors at further lower doses, with improved safety as well as reduced vector production costs, for gene therapy of a wide variety of diseases in humans.

INV69

Novel human brain organoids to study brain development and model diseases

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To study the unique traits of the developing human brain and to model associated diseases, the establishment of human-based model systems that can precisely capture *in vivo* like traits are essential. I will discuss recent work from our lab pursuing diverse approaches to improve physiological aspects of brain organoid models and discuss their applications in genetic disease and tumor modelling. Amongst others, these investigations led us to reveal an important role of morphogen signaling in shaping brain organoid tissue architecture, which in turn determines neural stem cell identity and brain expansion. Notably, the maintenance of tissue architecture is an essential feature to derive organoids derived from human fetal brain (FeBOs) requires. FeBOs derived from the early-to-mid neurogenesis period can be robustly expanded *in vitro*, akin the growth of the developing brain. We find that maintenance of tissue architecture promotes the generation of a tissue-like ECM niche. By genetic-based disease modelling resulting in interference with the ECM environment, we describe major alterations in brain organoid phenotypes, including reduced neural stem cell expansion and an altered cell fate switch. Finally, I will discuss how FeBOs constitute a novel platform for diverse bottom-up glioma modelling. In sum, environmental cues including architecture and ECM are active in shaping the developing human brain and its perturbation in disease results in perturbed developmental trajectories, which can be functionally studied in human organoid model systems.

INV70

Generation of fully human pluripotent stem cell-derived blood-brain barrier organoids for disease modeling and validation of neurotropic viruses

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The blood–brain barrier (BBB) is the highly selective interface between the systemic circulation and the neural parenchyma which is required for brain homeostasis and protection of the delicate neuronal environment. BBB is constituted by the intimate association between endothelial, mural and astroglial cells to constitute the neurovascular unit.

We established detailed procedures to differentiate human pluripotent-stem cells (hPSCs) into endothelial, mural and astroglial cells by combining an original combination of small molecule cocktails with the expression of lineage-specific transcription factors. To assemble the three cell types together we decided to generate organoids that recapitulate the spatial organization of the BBB either on transwell filters (taBBBOs) or as free-floating aggregates (ffBBBOs). We demonstrated that BBBOs display a tight and homogeneous endothelial cell layer with well-organized cell junctions clustering Glut1 and Occludin. BBBO functionality has been validated showing high values of trans-epithelial electric resistance (TEER) and impermeability to dyes with

high molecular mass. Single cell RNA-seq confirmed the fidelity of the lineage differentiation and the superior maturity of BBBO cells respect to conventional cell lines. Adeno-associated viruses (AAVs) and lentiviruses (LVs) failed to cross the BBBOs confirming their high impermeability. Thus, we employed BBBOs to determine the neurotropic potential of several novel engineered AAVs in the human setting comparing their relative BBBO crossing capacity and their intracellular trafficking pathways. BBBOs were also employed to decipher the pathological processes triggered by alpha-synuclein aggregation that leads to BBB leakage during Parkinson's disease progression. Altogether, the deployment of new differentiation protocols to generate and co-culture iPSC-derived endothelium, mural cells and astrocytes produced a robust in vitro neurovascular model with long-lasting stability and functions, offering a neat system for modeling diseases of the brain vasculature and testing new brain shuttling agents.

INV71

Nucleic Acid Delivery Systems for RNA Therapy and Genome Editing

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RNA nano formulations have received much attention for their role in bringing the covid pandemic under control. However, vaccines are only a small part of the broad potential use of RNA in human therapeutics. Here we describe our work developing nanoformulations for RNA therapy and genome editing. Libraries of degradable polymers and lipid-like materials have been synthesized, formulated and screened for their ability to delivery RNA payloads inside of cells. These nanoformulations facilitate in vivo delivery to a range of tissues, and can enable targeted gene suppression with siRNA, gene expression with mRNA, or even permanent genetic editing using the CRISPR/Cas9 system. We will describe the development and use of this technology, and describe their potential as therapies for a range of different diseases.

INV72

Mitochondrial DNA editing in vitro and in vivo

JS Kim [1]
1: National University of Singapore

Genome editing in nuclear, mitochondrial, and chloroplast DNA is broadly useful for biomedical research, medicine, and biotechnology. CRISPR systems, widely used for nuclear DNA editing, are not suitable for organellar DNA editing, due to the difficulty of guide RNA delivery into organelles. Mitochondrial DNA editing paves the way for disease modeling of mitochondrial genetic disorders in cell lines and animals and may offer treatment options for these diseases with unmet medical needs. I will present novel tools for A-to-G and C-to-T base editing of organellar DNA in vitro and in vivo.

Recent publications
1) Engineering TALE-linked deaminases to facilitate precision base editing in mitochondrial DNA.

Cell (2024)
2) Base editing of organellar DNA with programmable deaminases. Nat. Rev. Mol. Cell Biol.(2023)
3) Precision mitochondrial DNA editing with high-fidelity DddA-derived base editors. Nat. Biotechnol.(2023)
4) Targeted A-to-G base editing in human mitochondrial DNA with programmable deaminases. Cell (2022)
5) Chloroplast and mitochondrial DNA editing in plants. Nature Plants (2021)

INV73

Production of dual AAV vectors for the delivery of large genes

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AAV gene therapy, although successful in a number of settings, *in vitro* and *in vivo*, has been limited by transgene capacity. We have developed two AAV-based platforms to enable the delivery of large genes to tissues and cells *in vivo*; the first leveraging DNA recombination, named dual hybrid; and the second, utilising protein trans-splicing, named AAV-intein.

The dual hybrid platform uses two AAV vectors, each containing one half of an expression cassette encoding for a large therapeutic transgene and works at the cell nucleus level, recombining the two halves of the transgene back into a single one within the cell. This technology translates into an efficient recombination that generates therapeutically meaningful protein levels in animal models and it is a versatile approach.

The AAV-intein platform uses two AAV vectors, each containing an independent expression cassette encoding one of the 2 halves of a target protein flanked by short sequences, named as split inteins. It works by protein trans-splicing within the cell and has demonstrated a very efficient recombination to deliver therapeutically meaningful protein levels.

We are validating these platforms in two lead programs in Ophthalmology with a high unmet need: Usher Syndrome Type 1B associated retinitis pigmentosa (Usher1B), using the dual hybrid platform; and Stargardt disease, using our AAV intein-mediated platform. This would open-up the potential for treatment of additional diseases in other areas.

The focus of this presentation is on the development of the process and analytics and manufacture of the dual vectors to enable the supply for the above clinical programs. Data is presented on process and method establishment/development, optimisation and characterisation, as well as strategies for optimised generation of the dual vector product. Considerations on regulatory requirements for the current stage of development as well as for future planning up to registration are also discussed.

INV76

Messenger RNA therapy for liver inherited metabolic diseases

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RNA biotherapeutics represent >400 programs, >30 approved products and >60 billions USD in market capitalization. Messenger RNA (mRNA) encapsulated in lipid nanoparticles (LNP) is a leading non-viral gene therapy strategy, rapidly expanding for translation in vaccines and therapeutics, especially for rare genetic diseases. LNP-mRNA has been used for protein replacement or nuclease-mediated gene silencing, editing or integration.

>20 proof-of-concepts studies with mRNA replacement approach have been reported in LIMD since 2016. The first publication of interim safety and efficacy results in a phase I/II clinical trial for pediatric propionic acidemia (NCT04159103) was reported in April 2024. Four phase I/II clinical trials are currently recruiting for pediatric LIMD. mRNA-mediated gene silencing was successfully reported in 2021 in transthyretin amyloidosis (NCT04601051).

Here, we will review the rapid expansion of RNA biotherapeutics, the basics of this LNP-mRNA technology, its advantages/limitations compared to viral gene therapy, provide the latest updates on preclinical and clinical applications for LIMD, with a spotlight on our preclinical work on the urea cycle defect argininosuccinic aciduria.

INV77

Hematopoietic Stem Cell Gene Therapy for GM1-Gangliosidosis

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Lysosomal storage diseases (LSDs) include ~70 genetic diseases characterized by lysosomal dysfunction. Although individually rare, overall LSDs affect 1:5,000 live births. Most LSDs cause progressive neurodegeneration, lethal in early infancy. Treatment is usually symptomatic. Allogeneic Hematopoietic Stem/progenitor Cell transplantation (HSCT) may provide whole-body metabolic cross-correction by donor-derived cells acting as a local source of therapeutic enzyme, including in the central nervous (CNS). However, allogeneic HSCT is efficacious for few LSDs and is restricted by donor availability. Gene therapy (GT), i.e. transplantation of autologous HSCs genetically modified by lentiviral gene transfer, is an approved therapy in Europe and the USA for Metachromatic Leukodystrophy, a neurodegenerative LSD, and has proven successful in correcting Mucopolysaccharidosis (MPS) type I and IIIA, providing proof-of-principle for the efficacy of HSC gene therapy for neuro. LSDs. We designed and developed an HSC GT for a neuro. LSD caused by β-Galactosidase (β-gal) deficiency, *i.e.* GM1-gangliosidosis, based on the use of a lentiviral vector (LV) encoding the human or murine β-gal.

Transgenic β-gal was correctly processed, secreted and uptaken by target cells, and sustained phenotypical correction of GM1-patient derived primary fibroblasts. *In vitro*, both LVs may determine a dose-dependent and supra-physiological enzyme expression, with the murine enzyme showing a ~6-fold higher activity than the human enzyme. *In vivo*, GT was able to induce enzyme reconstitution in BM and brain, sustaining partial correction of neuromuscular phenotype and a significant survival increase. The murine enzyme showed a preferable correlation between enzymatic activity and vector copy number, with a significantly higher enzymatic activity *per* vector copy, supporting a superior therapeutic potential for GT applications. Phenotypic correction was coupled to a broad distribution of HSC-derived β-gal^{+} cells in the brain, associated with a partial clearance of storage material. These observations supported the hypothesis of a limited cross-correction potential of the wild-type β-gal enzyme associated to the classical mechanism of enzyme secretion/uptake. We are currently developing novel hyperfunctional human β-gal isoforms by enzyme evolution, to further increase feasibility, efficacy and reduce dose and cost of the GT. Novel enzymes will be evaluated in relevant *in vitro* and *in vivo* preclinical models, including human brain organoids and the GM1 mouse model

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INV78

Towards production of nucleic acid nanomedicines at the bedside

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Nucleic acid therapeutics offer promising solutions for treating a wide range of conditions, especially those linked to protein anomalies, which are the root cause of many human diseases. These therapies provide high precision and adaptability, making them suitable for conditions like cancer, rare "orphan" diseases, and vaccine development, as seen with the rapid creation of mRNA vaccines for COVID-19 variants. However, despite their potential for personalized care, they face challenges such as pharmaceutical barriers, limited commercial incentives for orphan diseases, approval and reimbursement difficulties, and high costs for health systems.

One major benefit of nucleic acid therapeutics is their suitability for personalized production in hospital pharmacies. Since these therapies are essentially the same molecules with slight variations, they can be tailored to individual needs, which is particularly useful for orphan diseases. Nanomedicine platforms, such as lipid nanoparticles, help by encapsulating these therapies and delivering them efficiently to their target cells. These platforms can be manufactured on a small scale, offering the flexibility to treat a variety of conditions.

The NANOSPRESSO model combines nucleic acid therapeutics with nanomedicine delivery systems, allowing for the rapid, personalized production of treatments at the point of care. This approach could make these innovative therapies more accessible, especially for orphan diseases and in low-income regions. To achieve this, collaboration across multiple sectors is vital. Scientists, engineers, healthcare providers, policymakers, and others must work together to ensure these therapies are financially sustainable and accessible to all.

INV79

Utilizing extracellular vesicles for RNA transport and therapeutic applications

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RNA-based therapeutics hold great potential for treating various diseases by either silencing pathological genes or expressing therapeutic proteins. Lipid nanoparticles (LNPs) are currently used to transport functional mRNAs, such as in COVID-19 mRNA vaccines. However, the fate of LNP-delivered mRNA after endocytosis and escape from the autophagy-lysosomal pathway remains unclear. To address this, we investigated the delivery and intracellular trafficking of various exogenous mRNAs using LNPs, uncovering for the first time a link between LNP-mRNA endocytosis and its subsequent packaging into extracellular vesicles (EVs).

EVs are a heterogeneous population of nano- and micro-sized vesicles, including exosomes, secreted by nearly every cell type studied so far. They transport a variety of biomolecules, including RNAs, proteins, and lipids, between cells. Our study revealed that portions of the LNP-delivered mRNA, along with the ionizable lipid components of the LNPs that had not been degraded, were incorporated into EVs and secreted from cells. Interestingly, these EVs contained one mole of ionizable lipids per mole of mRNA nucleotides, compared to the three-to-one molar ratio found in LNPs. Importantly, these EVs were capable of protecting the LNP-delivered mRNA during in vivo delivery and facilitated the production of human proteins in mice, with detectable levels in both plasma and various organs. This finding suggests that EVs can act as vehicles for spreading LNP-delivered mRNA between cells.

To further explore this phenomenon, we investigated the delivery of VEGF mRNA (encoding Vascular Endothelial Growth Factor A) to cells via LNPs. Our results demonstrated that LNPs were rapidly taken up by cells, and translation of the exogenous mRNA began almost immediately after cellular entry. This rapid uptake and translation process was confirmed in three different cell types: cardiac progenitor cells (CPCs), human umbilical vein endothelial cells (HUVECs), and an epithelial cell line (HTB177). Following LNP-mediated delivery of VEGF mRNA to these cells, we observed that a fraction of the internalized LNP mRNA was also secreted via EVs derived from each cell type. Additionally, we examined whether the expression of other transcripts in these EVs changed after their parent cells were treated with LNPs containing VEGF mRNA. Our findings showed that, in addition to the presence of VEGF mRNA in these EVs, several other genes had altered expression profiles. Notably, many of these changes were associated with angiogenesis, indicating that the EVs might influence vascular formation pathways.

To assess the functional impact of these EVs, we compared their ability to promote angiogenesis (endothelial vascular network formation) with that of LNPs delivering an equivalent amount of VEGF-A mRNA. While LNPs were more efficient in delivering VEGF mRNA and resulted in the highest levels of VEGF protein production in vitro, CPC-derived EVs were unexpectedly the most effective in promoting angiogenesis, even though they produced approximately threefold less VEGF protein than LNPs. This result underscores the potential of EVs to enhance biological processes like angiogenesis, independent of their relative efficiency in mRNA delivery and protein production.

INV83

Modelling neurological disorders to advance gene therapies

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Neurodevelopmental disorders comprise a heterogeneous group of conditions characterised by developmental delay, usually associated with other neurological and systemic co-morbidities. Studying these disorders can be challenging, given the relative inaccessibility of brain tissue and paucity of animal models that faithfully recapitulate human phenotype. iPSC-derived systems provide a new avenue for both studying disease mechanisms and developing novel therapies in a humanised model. As an adjunct to animal models, such iPSC-derived neuronal models can provide important proof-of-concept data regarding the safety and efficacy of genetic therapies. In this talk, I will discuss the growing utility of iPSC-derived neuronal models in tandem with animal models for developing new genetic therapies.

INV85

Early clinical trials in developing a stem cell-based therapy in Parkinson's disease

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In Parkinson's disease (PD) specific regional cell loss of dopamine neurons of the substantia nigra results in debilitating clinical signs and symptoms. Dopaminergic inputs are lost progressively to the putamen more than caudate, altering motor control pathways and resulting in bradykinesia, rigidity, and other cardinal signs of PD that impact upon daily function and quality of life. This makes a cell replacement strategy highly compelling. With more than two decades of advances in stem cell technology, combined with sophisticated differentiation protocols to produce authentic A9 dopamine neurons and progenitors, a number of cell-based interventions to replace loss of striatal dopamine have been successful in preclinical models of PD. These efforts include working to optimize cell products, surgical delivery, and where applicable ancillary immunosuppressive regimens. Approaches in current clinical trials based upon surgical transplant of dopamine neuron progenitors derived from autologous and allogeneic iPSCs, and allogeneic hESCs will be critically

discussed. While early clinical trials results are focused upon safety and tolerability, available data on cell survival and clinical effects will be presented, with a focus on what this means for next steps.

INV86

In vivo selection in cell and gene therapy

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The success of hematopoietic stem cell transplantation and gene therapies has been wholly dependent on the development of conditioning regimens that provide a selective advantage for therapeutic stem cells. Despite this clear example of success similar conditioning/selection regimens have not yet been developed for other tissues/organs. In this lecture, we will briefly review examples of natural in vivo selection of immune cells, skin cells and hepatocytes and discuss their relevance to gene therapy. Focussing on the liver, novel systems designed to engineer in vivo selection of therapeutic cells in the liver will be shown.

Experiments of nature in rare genetic diseases have illustrated that healthy hepatocytes may have a selective advantage, outcompete diseased cells and result in extensive liver repopulation. This observation has given rise to the concept of therapeutic liver repopulation by providing an engineered selective advantage to a subpopulation of beneficial hepatocytes. Recently, we developed a universal hepatocyte selection system that relies on the widely used fever medicine acetaminophen (Paracetamol) to safely achieve therapeutic levels of hepatocyte expansion. In vivo selection can greatly enhance the efficiency of both gene and cell transplantation therapies for hepatic diseases.

LW01

How to get published -- in *Human Gene Therapy* (and elsewhere)!

K Davies [1] Thomas Gallagher [2]
1: Executive Editor, The CRISPR Journal 2:Managing Editor, Human Gene Therapy

Success in academic research is measured to a large extent by one's publication record. The maxim "publish or perish" is as true now as it ever has been. But junior researchers at the grad student and postdoc level may feel confused or intimidated by the process of science publishing and uncertain as to the rules of engagement with the editorial teams at their favorite peer-review journals.

In this ESGCT student workshop, two professional editors affiliated with *Human Gene Therapy* and its parent publisher seek to demystify the sometimes arcane and mysterious world of science publishing. **Kevin Davies**, the founding editor of *Nature Genetics* and Executive Editor of The *CRISPR Journal*, will share some personal insights from 30+ years as a journal editor and publisher. **Tom Gallagher**, the Managing Editor of *Human Gene Therapy*, will offer insights from the journal's perspective of the peer review process and advice on how to increase your chances of publication.

Together, Kevin and Tom will offer advice and suggestions for ways that young scientists can improve their chances of publication and work with journal editorial teams. They will also reveal some of the perks of working in science publishing and communication for those who might be contemplating a career beyond the bench.

Oral Presentations

OR001

Dose escalation studies in mice and NHPs confirm biodistribution, target engagement and safety of AAV9-miR871 and support its translation for the treatment of CMT1A neuropathy

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CMT1A is the most common inherited demyelinating peripheral neuropathy, resulting from the duplication of *PMP22* gene that leads to demyelination, secondary axonal loss and progressive disabilities. We previously developed and validated an AAV9 vector expressing the engineered *PMP22*-targeting miR871 that showed therapeutic effects in CMT1A mice after lumbar intrathecal injection. To demonstrate the safety and translational potential of this approach, we performed further dose escalation studies in CMT1A mice and in non-human primates (NHPs) using a clinical stage optimized miR expression cassette.

CMT1A mice were intrathecally injected with different doses of AAV9-U6-miR871 (1e11, 2e11, 5e11, 1e12 vg/mouse) and examined with VGCN, histopathological, CD markers, RNAscope Plus, RNA and protein analysis at 6 weeks post-injection as well as with behavioural, electrophysiological, morphometric and blood NF-L level analysis at 4 months post-injection. Treated mice showed a dose-depended transduction of all examined neural and peripheral tissues. Histopathology analysis showed no article related findings in any of the examined tissues except for a mild dose-depended liver pathology. CD markers, RNA and protein analysis, as well as behavioural, electrophysiological, morphometric and NF-L readouts suggested 5e11 vg/mouse as the most effective dose for improving CMT1A neuropathy in mice. Single cell resolution analysis with RNAscope Plus showed that more than 50% of Schwann cells express miR871, leading to a uniform moderate silencing without loss of *PMP22/Pmp22* gene expression.

NHPs were intrathecally injected with 2 doses of AAV9-U6-miR871, 6e13 or 1.2e14 vg/NHP, extrapolated from the therapeutically effective and safe doses of 2e11 and 5e11 vg/mouse, based on CSF volumes, or with PBS, and analysed at 6- or 12-weeks post-injection. NHPs were longitudinally examined with clinical, neurological, blood and urine tests. At the final time point, animals underwent ophthalmic testing, electrocardiogram, nerve conduction studies, histopathological and molecular analysis. NHP results confirmed the scale up potential of lumbar intrathecal injection with both vector doses resulting in efficient transduction of proximal and distal PNS tissues and stable expression of miR871. Target engagement was demonstrated by efficient silencing of NHP *Pmp22* RNA and protein levels across PNS tissues at 6- and 12-weeks

post injection, with the 1.2e14 vg dose being more robust. In-life observations and post-mortem analysis confirmed the safety of AAV9-U6-miR871 with NHPs showing no HNPP-like phenotypes, no behavioural abnormalities, no clinical chemistry alterations, and no impairment of tibial nerve conduction studies. Minor, subclinical inflammation was found in DRGs of some treated animals.

This study provides for the first-time detailed dose escalation data of a gene silencing approach in CMT1A mice and NHPs. Murine and NHP data suggested 5e11 vg/mouse and 1.2e14 vg/NHP as the safest and most efficient dose of AAV9-miR871 to treat CMT1A. Detailed biodistribution and target engagement analysis confirms efficient transduction of Schwann cells throughout the PNS of rodents and NHPs, and promising safety data in NHPs of an intrathecally-delivered AAV9 vector to silence overexpressed PMP22. Collectively, these results support the scale-up potential and further clinical development of AAV9-U6-miR871 for CMT1A treatment while also providing valuable information for AAV9-mediated gene therapies for other demyelinating neuropathies as well.

OR002

Developing a screening platform for AAV tropism in iPSC-derived neural cells

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Adeno-associated viruses (AAVs) have emerged as a promising tool for in vivo gene delivery due to their diverse tissue preferences, robust transduction capabilities, low immunogenicity, and cargo capacity. While extensively studied in animal models, the translation of AAV tropism from tissues to cultured primary cells and human induced pluripotent stem cell (hiPSC)-derived models remains challenging. The development of AAVs with high tropism for these cultured cell types would be a valuable tool, as hiPSC-derived neural cell types offer the unique opportunity to study the pathogenesis of neurodegenerative diseases in a human model system and in the affected cell type. This is underscored by the fact that transfection and/or transduction of fully differentiated cells, especially of the CNS, using classical methods still poses major obstacles.

To address these challenges, we aim to develop a high-throughput screening platform for the assessment of AAV tropism on hiPSC-derived neural cells. The automated confocal imaging and analysis capabilities of this platform will enable the screening of various AAV serotypes at different concentrations, allowing the parallel assessment of serotype-specific transduction efficacy and toxicity.

In a proof-of concept study, we have analyzed the transduction efficacy of 18 different AAV serotypes in iPSC-derived cortical neurons, dopaminergic neurons and NGN2 neurons. Several AAV serotypes were identified that have a high transduction efficacy throughout all cell types tested without any obvious neurotoxic effects. The implementation of more cell types including glia and vascular cells will further improve the versatility of this platform and provide insights on cell type preferences.

The objective of this study is to develop an accessible and scalable method to test established and novel AAV capsids on different hiPSC-derived neural cell types, with the aim of identifying highly efficient and selective capsid variants. These could serve as a versatile tool to study disease

pathology and genetic intervention in a standardized, scalable human model system. The results from screenable 2D monocultures lay the foundation for the implementation of AAV in complex model systems such as co-culture systems and brain organoids. Ultimately, this platform has the potential to identify AAV serotypes that are optimal for delivering genomic cargo to hiPSC-based disease models, thereby advancing our understanding of neurodegenerative diseases in the human context and facilitating potential therapeutic interventions.

OR003

A human, multi-lineage and multi-organoid platform to assess tropism, toxicity and efficacy of neuromuscular gene therapy vectors

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Neuromuscular disorders, including muscular dystrophies, are severe, inherited disorders characterised by muscle wasting, limited mobility and premature death. Although no curative treatment is currently available for muscular dystrophies, adeno-associated virus- (AAV) mediated gene therapy is emerging as a promising therapeutic strategy. However, clinical success of AAVs in muscular dystrophies remains limited, due to modest level of efficacy or severe adverse events, often not predicted by current pre-clinical models. Moreover, development of new AAV variants with safer and more efficacious profile is hindered by species-specific challenges, with an increasing number of cases showing discrepancy between rodent and primate data. These limitations could be addressed by robust, human(ised) models predicting cell-/tissue-specificity, toxicity and efficacy during AAV development. To this aim, we describe here a novel *in vitro / quasi-vivo* platform based upon human 3D cultures and organoids engineered to assess tropism, toxicity and efficacy of AAVs, with pre-clinical or clinical relevance for neuromuscular disorders. We first assessed if our 3D, human induced pluripotent stem cell- (iPSC) based platform to engineer skeletal muscle constructs would be compatible with direct *in vitro* AAV transduction and then explored AAV dose-response by transgene expression dynamics of several natural AAV serotypes. Morphological and molecular assessment of a fluorescent reporter transgene expression demonstrated dose-dependent signal increase in transduced engineered skeletal muscles, alongside deep tissue penetration of AAV particles in the 3D tissues. Moreover, post-transduction live-imaging data indicated that a 14-day time course is sufficient to determine differential performances in terms of peak reporter expression amongst several different natural serotypes. Thereafter, we harnessed the multi-cellular and multi-lineage nature of our 3D platform to investigate tropism of natural and recombinant AAV serotypes (rAAVs) in engineered muscles containing isogenic myofibers and motoneurons. Analyses of transduced constructs indicate preferential tropism of neuronal-specific rAAVs in SMI32-positive human motoneurons compared to myosin-positive myotubes, in keeping with conventional (more laborious, expensive and ethically challenging) *in vivo* assays performed in animal models. Additionally, we also validated that neuronal-specific rAAVs outperformed natural serotypes in transducing motoneurons in human 3D cultures. We then expanded the resolution of this platform beyond

skeletal muscle, establishing a multi organ(oid) system to perform similar assays in other relevant AAV on- and off-target tissues, such as the heart and the liver to study cardiac tropism and hepatotoxicity. Finally, we provide evidence of the potential for this platform to assess efficacy of therapeutically relevant transgenes for Duchenne muscular dystrophy by testing functional properties of target tissues, such as amelioration of muscle contractility. Taken together, these data provide a significant step forward towards preclinical development and screening of viral vectors in a human-specific context to improve gene therapy outcomes in children with muscular dystrophies and beyond.

OR004

Understanding the involvement of AAVR in AAV entry and transduction of the central nervous system

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Vectors based on adeno-associated viruses (AAV) are essential tools in gene therapy, favoured for their safety profile and delivery capabilities. However, their application in targeting the disorders of the central nervous system (CNS) is hampered by the challenge of crossing the blood-brain barrier (BBB). Recognizing the need for enhanced CNS delivery, our study focused on understanding and overcoming these barriers through targeted capsid modifications.

To elucidate capsid structural elements essential for BBB crossing, we employed a domain-swapping strategy between our previously published AAV-BBB6 (Drouyer et al., Molecular Therapy 2024), which is able to cross the mouse BBB and its parental strain AAV1, which lacks the ability to cross the BBB. This comparative approach revealed that two amino acids, N498del and T502A in VR-V, were essential for this characteristic of AAV-BBB6. The deletion and substitution of these residues, respectively, in AAV1 markedly improved its ability to traverse the BBB. These findings were quantitatively supported by enhanced vector copy numbers in the neuronal cells of treated mice, showing a 50% increase in transduction efficiency over the parental AAV1.

Leveraging insights from previous studies, we incorporated the N498del and T502A mutations, collectively termed C3, into AAV6. This mirrored the enhanced BBB penetration and CNS delivery observed with AAV-BBB6, suggesting a potentially universal applicability of C3 in enhancing CNS transduction across different AAV serotypes. Subsequent experiments using another proprietary capsid, AAV-BBB37, and its variant AAV-BBB37-C3 in mice demonstrated a 70-fold increase in vector copy number for AAV-BBB37-C3 compared to AAV-BBB37. However, no significant increase at the transgene expression level was observed. Additionally, *in vitro* binding assays revealed an 80-fold increase in AAV-BBB37-C3's binding capacity to Ly6a-expressing cells compared to its parental counterpart yet failed to enhance neuronal expression, suggesting that some other residue(s) in AAV-BBB37 is essential for functional transduction of brain parenchymal cells.

Sequence analysis identified a unique mutation within VR-I of AAV-BBB37: a threonine deletion at position 266 (T266del/T265del based on AAV1 sequence). Incorporating T265del into AAV1-C3, resulting in AAV1-C3-T265del, led to a significant shift in vector tropism from brain parenchymal cells to vascular cells. *In silico* modelling and subsequent pull-down assays confirmed that this mutation significantly impairs the interaction between the capsid and the AAV receptor

(AAVR), while maintaining interaction with LY6A. This suggests a transformative shift in vector behaviour, with the threonine deletion within VR-I prompting a switch from transcytosis to functional transduction of brain endothelial cells.

Our *in vitro* and *in vivo* assays reveal that N498del and T502A mutations are essential for effective BBB penetration, while the T265del mutation critically redirects vector tropism from brain parenchymal cells to vascular cells. These findings illustrate that capsid tropism is not solely dependent on BBB receptor binding and that modulating the strength of AAVR interaction can significantly alter capsid functionality. By identifying and fine-tuning these key capsid modifications, our findings contribute to the ongoing efforts in bioengineering AAV capsids, enhancing the potential for more effective vectors for CNS-targeted gene therapies.

OR005

The genomic architecture of 24 carcinogenic recombinant-AAV integrations in mice: Implications for human gene therapy safety

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Hepatocellular carcinoma (HCC) in mice exposed to recombinant adeno-associated viral (rAAV) vectors was first reported over two decades ago. More recent reports of an association between human HCCs and wildtype AAV infections, rAAV integrations with clonal expansion in hemophilic dogs, and the formation of an HCC in patient following rAAV treatment for hemophilia continues to raise concerns about the possibility of rAAV integrations causing insertional mutagenesis in humans. Elucidating the mechanisms by which rAAV causes HCC in mice after integration will provide critical insights that might help mitigate potential toxicity after rAAV gene therapy in humans. To date, the complete genomic characterization of rAAV associated HCCs has been limited to the capture of a single ITR junction, with PCR based genomic walking across integrations attempted by several groups in the past without success. We used LAM PCR, locus specific amplification, Sanger sequencing, and linked-reads and a long-read sequencing platforms (10x Genomics and Oxford Nanopore) to study HCCs from a historic cohort of 33 MMA mice and unaffected control littermates treated with a variety of single stranded therapeutic and reporter rAAV vectors (R. Chandler JCI 2015, PMCID:4319425). We detected rAAV sequence in 24 murine HCCs: 17 captured the entire integration and 7 captured partial rAAV integrations. All 24 rAAV integrations mapped to the *Rian* locus. The 17 rAAV integration events completely sequenced ranged in size from 255 to 4,535 base pairs; 15 contained truncated rAAV genomes less than 1.3 kilobases in size. Only 3 of the integrations contained the transgene coding sequence with 2 of these events having a majority of the packaged rAAV genome intact. Substantially rearranged ITRs were recovered in all but two of the integrations. One rAAV integration contained the origin of replication (*ori*) from the plasmid used to package the rAAV in cis with a truncated vector genome. The only common element in all 24 rAAV integrations was rAAV enhancer. Concatemers that underwent intramolecular recombination and subsequent deletion are predicted as the in vivo precursor(s) of some integrations, and the identification of an event that contains a reverse packaged vector plasmid *ori* suggest that future studies should focus on longitudinal assessment of integration events in animal models, with an expansion to query all elements of plasmids used to produce rAAV as possible candidates for unwanted integrations. Interesting, all integration containing reads were associated with cis DNA hypomethylation, while

the corresponding reads at the empty sites were universally hypermethylated. RNAseq confirmed that integration-associated DNA hypomethylation was associated with microRNA over-expression at the *Rian* locus and identified a perturbation of the H19 axis in some samples. Our findings support a model where rAAV-mediated tumorigenesis is caused by enhancer insertion, with subsequent dysregulated transcription. Because all the genotoxic integrations contained enhancers present in rAAV vectors that have been administered to thousands of patients, our studies mandate the design of safer vectors devoid of strong-enhancer elements for human translation and highlight the need to define the mechanism(s) by which rAAVs integrate and can cause cancer.

OR006

Transcriptional findings of unfolded protein response/integrated stress response in the liver of non-human primates subjected to high doses of recombinant AAV vector

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Adeno-associated viral vector (AAV)-mediated gene therapy is the leading *in vivo* gene delivery platform. However, reports of serious adverse events after high AAV dose administration, such as liver toxicity that manifests as elevated liver enzymes, liver failure and even death, have raised concerns. Thus, understanding the underlying mechanisms of hepatotoxicity has become crucial. Recombinant AAVs have been shown to induce endoplasmic reticulum (ER) stress by overwhelming the ER protein folding capacity and to promote the activation of the unfolded protein response (UPR)/integrated stress response (ISR). The UPR/ISR are homeostatic mechanisms that monitor and maintain cellular homeostasis, but can induce apoptotic cell death if stress remains unresolved. Since the UPR/ISR drive a well-established transcriptional response to cope with stress, the goal of this study is to analyse the transcriptomic signature of these pathways in RNA obtained from the liver of male and female non-human primates (NHP) receiving doses over 2×10^{13} vg/kg that can cause severe acute hepatotoxicity. In one study, animals were dosed with 2×10^{13}, 5×10^{13} and 1×10^{14} vg/kg of AAV9PHP.B-CBh-SMN1, and were sacrificed on day 4 after dosing (Palazzi et al., 2021). Gene Set Enrichment Analysis (GSEA) in the liver of NHPs dosed with $>5\times10^{13}$ vg/kg revealed enrichment of genes related to cytotoxicity and genotoxicity. Loss of enrichment in genes related to liver metabolic function was also observed. Furthermore, significant upregulation of UPR/ISR related genes like PERK, GADD34, ATF4, and CHOP was detected in both male and female NHPs in a dose dependent manner. Rats injected with the same vector and doses did not show liver injury neither UPR/ISR, most likely due to significantly lower transgene expression. We performed GSEA analysis on a published single nucleus RNAseq data set from another study in which NHPs received 7.5×10^{13} and 1×10^{14} vg/kg of AAV-PHP.eB.EGFP, and were sacrificed 3 to 6 days post injection with clear signs of hepatocyte injury (Hordeaux et al., 2024). Our analysis showed a positive enrichment of genes related to autophagy, protein stability and intrinsic apoptosis signalling pathway, and negative enrichment of genes related to metabolic pathways, confirming the original publication analysis.

ATF4-induced genes were also slightly upregulated, suggesting UPR/ISR activation. In conclusion, transcriptomic analyses from two independent studies in NHPs showed UPR/ISR activation induced by hepatotoxic AAV doses. Next, we will evaluate if modulation of UPR/ISR response activated by high dose of AAV has a beneficial or detrimental effect on AAV-mediate hepatotoxicity.

OR007

In vivo lentiviral gene therapy for distal urea cycle defects

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The urea cycle enables nitrogen waste and clearance of neurotoxic ammonia. Argininosuccinate synthase (ASS) and argininosuccinate lyase (ASL) are cytoplasmic enzymes involved in the distal part of the urea cycle pathway. Argininosuccinic aciduria (ASA) and Citrullinaemia type 1 (CTLN1), caused by ASL and ASS deficiencies respectively, are the second and third most common urea cycle defects. Patients present either neonatal- or late-onset hyperammonaemia, causing cerebral oedema and ultimately death if untreated, associated with a high risk of lifelong severe neurodisability. Liver transplantation is the only cure but is invasive and requires lifetime immunosuppression. We aimed to test *in vivo* lentiviral gene therapy in neonatal ASL- and ASS-deficient mice. ASL-deficient ($Asl^{Neo/Neo}$) pups received intravenous injection of lentiviral vector encoding codon-optimised human *ASL* (LV.*coASL*) (n=8) versus GFP (n=8), both at a dose of 4E10TU/kg . LV.*coASL*-injected animals survived the 12 weeks experiment whilst control mice died within 4 weeks ($p<0.001$). Growth ($p<0.01$), fur coat pattern, ammonia ($p<0.001$), arginosuccinate ($p<0.001$), citrulline ($p<0.01$), and orotate ($p<0.05$) were normalised to wild-type levels. Significantly increased ASL expression (+300%; $p<0.01$) and activity (+30%; $p<0.05$) were observed in treated $Asl^{Neo/Neo}$ livers compared to controls. Lentiviral vectors present long-term transgene expression due to their ability to integrate in the host genome. We conducted safety studies to identify genotoxic events driven by the LV.*cohASL* in 20 control mice. No significant difference was shown for survival and liver/body ratio. Pathology analysis and dissection of the liver, spleen and main organs showed no lesion. Lentiviral biodistribution was predominant in the liver and to a significantly lower extent in the spleen and lungs. This positive outcome was further confirmed by the integration site analysis performed on murine livers at 9 months after *in vivo* transduction. To maximise safety and efficacy, we investigated macrophage inhibition by pre-treatment with systemic clodronate before lentiviral vector administration. This resulted in significant increased liver transduction *in vivo* ($p<0.01$). This approach was applied to treat ASS-deficient ($Ass^{Fold/Fold}$) mice. $Ass^{Fold/Fold}$ pups (n=6) received intravenous injection of lentiviral vector encoding codon-optimised human *ASS* (LV.*coASS*) at 3E10TU/kg preceded by macrophage inhibition. Compared to LV.*GFP*-injected controls (n=4), LV.*coASS*-injected mice demonstrated significantly improved survival ($p<0.05$), phenotypic improvement but no significant changes in biomarkers despite the substantial liver transduction of the vector. We are investigating additional strategies to maximize the therapeutic potential of LV vector in ASS-deficient mice.

Overall, these studies support proof of concept of safety and efficacy of *in vivo* lentiviral gene therapy for distal urea cycle diseases.

OR008

Comparison of different lentiviral vectors allows the selection of an efficient and safe vector design for *in vivo* gene therapy of homozygous familial hypercholesterolemia

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Homozygous familial hypercholesterolemia (HoFH) is a severe rare early-onset inherited disease, mostly due to mutations in the low-density lipoprotein receptor (LDLR)-encoding gene. The disease is characterized by high circulating LDL cholesterol (LDL-c), due to impairment in its clearance by LDLR. Patients rapidly develop atherosclerosis and cardiovascular disease. Available treatments do not normalize LDL-c. *In vivo* gene transfer to hepatocytes may rescue LDLR expression upon a single intravenous (i.v.) administration. Lentiviral vectors (LV) may be used for this purpose since they integrate into the genome of target cells and thus are maintained upon cell proliferation in liver growth. Therefore, LV may be applied to pediatric patients at first disease stage. LV are typically pseudotyped with vesicular stomatitis virus glycoprotein (VSV.G). However, VSV.G-LV have been shown to interact with LDLR and LDLR family members to access target cells. Indeed, when producing VSV.G-LV expressing LDLR transgene, we obtained very low titers, likely due to interaction between VSV.G and LDLR overexpressed by producer cells and subsequent re-infection during LV production. To overcome this issue, we abolished LDLR expression during production, by placing the LDLR sequence in an antisense orientation with respect to LV genome transcription, and achieved complete rescue of productivity. We produced LV expressing LDLR under the control of a strong hepatocyte-specific promoter (enhanced transthyretin, ET) and administered ET.LDLR-LV to juvenile Ldlr-/- mice. We observed normalization of blood LDL-c. We stressed the mice with three months of Western Diet, without detecting any atherosclerosis in treated mice, which was marked in Ldlr-/- controls. We then tested safety, by following ET.LDLR-LV treated mice for one year. While we confirmed the normalization of LDL-c, we found adenomas in several treated mice by histopathology. This outcome could be due to the antisense-oriented LV configuration. To rescue the yield of LV expressing LDLR in sense-orientation, we added an LDLR inhibitor during LV production. We treated juvenile mice with these ET.LDLR-LV and followed them for one year. We confirmed normalization of LDL-c but, even in this case, found lesions in some of the treated mice. We characterized them by LV integration site analysis and found a recurrent integration in the FRK gene, a known hepatic oncogene. We detected aberrant transcripts generated by the fusion of part of the transgene with FRK, suggesting a “promoter insertion” mechanism of genotoxicity. The genotoxicity may be due to a combination of the strength of ET promoter and increased vacuolation observed in LV-treated mice long-term. To improve this outcome, we tested a weaker alpha-1-antitrypsin (HAAT) promoter. At matched LV doses, HAAT was ~7-fold lower *in vivo*. Interestingly, when tested in Ldlr-/- mice, we found similar therapeutic efficacy of LV equipped with the two promoters. Importantly, in the safety experiment of one-year follow-up, no microscopic abnormalities were detected by histopathology, in addition to full efficacy. Here, we

show, for the first time, to our knowledge, the efficacy and safety of a liver-directed LV gene therapy for HoFH. These data provide the basis for further development towards clinical translation.

OR010

Severe inflammation and lineage skewing are associated with poor engraftment of engineered hematopoietic stem cells in patients with sickle cell disease

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Sickle cell disease (SCD) is one of the most common genetic diseases in the world caused by a single point of mutation in the beta-globin gene (*HBB*). This mutation leads to hemoglobin polymerization and red blood cell sickling, resulting in anemia and vaso-occlusive crises. A definitive cure for SCD patients is represented by hematopoietic stem and progenitor cell (HSPC) transplantation. Still, gene therapy (GT) is a promising treatment for patients lacking an HLA-compatible donor.

A Phase I/II clinical trial based on the DREPAGLOBE lentiviral vector, driving erythroid-specific expression of an anti-sickling globin, has been conducted at the Necker hospital (NCT03964792). Two out of 4 patients showed stable engraftment of the gene-modified HSPCs with a clinical benefit (P1 and P2). In contrast, the other two patients (P3 and P4) showed a progressive loss of corrected cells over time. To gain insights into factors that may explain the variable levels of engraftment of gene-corrected cells in the 4 patients, we performed a transcriptomic analysis of plerixafor-mobilized HSPC from the treated SCD patients or healthy donors (HD). Single-cell RNAseq analysis combined with multiparameter flow cytometry analysis allowed us to analyze the most immature HSC compartment as well as the more mature progenitor populations. Interestingly, SCD HSCs showed an exacerbated inflammatory signature (via IL-1 or interferon signaling pathways) compared to healthy donors. This was particularly evident for P4, who presented a defective long-term engraftment, and was accompanied by a myeloid lineage bias of HSCs. P3 (with poor engraftment of corrected cells) presented the activation of an aberrant Megakaryocyte (Mk) program as shown by an abnormal population of cells with a mixed HSC/MkP signature, CD71-low HSC phenotype, the increase of Mk progenitors and the presence of an abnormal erythrocytic/megakaryocytic progenitor population (expressing the mature CD71 erythroid and CD41 megakaryocyte markers). In conclusion, this study shows transcriptomic alterations in the HSC compartment and reveals an HSC bias toward Mk or myeloid progenitors respectively in P3 and P4 patients.

Altogether these results suggest that the chronic inflammation in SCD induces transcriptomic alterations at the HSC level. Chronic inflammation can lead to premature aging of HSCs, which

are biased towards the myeloid or megakaryocytic lineages, thus potentially impairing the long-term engraftment in two out of 4 patients. The pathways found to be dysregulated in our study are potential pharmacological targets for correction of the SCD HSC phenotype (e.g., anti-inflammatory drugs). Targeted treatments could be administered before GT to protect HSC functionality and achieve a successful clinical outcome in SCD patients. In addition, the identification of predictive biomarkers would allow HSCs to be screened using targeted RNA sequencing. This could allow stratification of patients with a low inflammatory signature score (who could be treated safely with GT) from those with a high inflammatory signature score (who could be pretreated with anti-inflammatory drugs).

OR011

Predicting the risk of insertional mutagenesis with SAGA-Q

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Lentiviral gene transfer is a promising therapy for rare disorders with otherwise limited treatment options. Safety concerns must be addressed and discussed with regulators to achieve the vital step from preclinical tests to clinical translation. In particular, the risk of insertional mutagenesis can be hard to measure. Only one predictive animal model and few *in vitro* assays exist. The surrogate assay for genotoxicity assessment (SAGA) recently gained attention for its ability to measure the potential of new vector designs to dysregulate a gene set linked to the insertional transformation of mouse hematopoietic stem and progenitor cells. SAGA depends on 11 genes to distinguish mutagenic from safer vectors. Even though the microarray-based readout is more sensitive and robust compared to previous technologies, other laboratories rarely adopt the workflow for two reasons. First, no comprehensive paper has described the critical steps of SAGA's underlying cell culture protocol, the in vitro immortalization assay (IVIM). The establishment of this technique with the associated controls is time-consuming and expensive. Second, the microarray technology seems outdated. Even though the design is described and available, few research institutes anymore possess a microarray reader. To address these issues, we developed the ddPCR-based SAGA-Q. Here, we describe how an optimized set of genes can predict the risk of insertional mutagenesis with a technology readily accessible in most labs.

OR012

A mouse model of genotoxicity in HSPC gene therapy to investigate the persistence of senescent cells

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Gene therapy (GT) product approval requires long-term safety studies to characterize the genotoxic potential, or lack thereof, of an integrating vector. Pre-clinical and clinical studies indicate that retroviral vectors used in hematopoietic stem and progenitor cell (HSPC) GT may integrate near oncogenes, leading to their activation and potentially causing tumorigenesis. However, adverse effects post insertional mutagenesis may occur before tumorigenesis. We previously showed that oncogene (*BRAFV600E*) expression in human HSPCs triggers another cellular mechanism called senescence, resulting in permanent cell cycle arrest and the onset of the senescence-associated secretory phenotype (SASP). SASP consists of the secretion of pro-inflammatory cytokines for the activation of immune cells, favoring the clearance of SCs. Yet, inefficient clearance of SCs may result in chronic inflammation, associated with poor immune function, increased risk of malignancy, and accelerated aging. Therefore, understanding the factors impacting SC persistence is crucial for enhancing GT safety and developing therapies to clear SCs.

We investigated the impact of the immunological background on the persistence of SCs, with the intent of identifying early genotoxicity biomarkers. To do so, we generated two mouse models through the transplantation of mouse HSPCs expressing BrafV600E in immune-deficient (NSG) or immune-competent (WT) mice.

In NSG mice (n=12), BrafV600E-expressing HSPCs caused dose-dependent lethality (Median = 36 days), while 40% of WT mice (n=7) survived up to 500 days and eliminated SCs. Both models showed histiocytosis and impaired hematopoiesis, specifically of lymphoid cells. Control and BrafV600E groups in NSG mice displayed higher levels of immune-suppressive cytokines, such as IL-4, IL-9, and IL-10, the latter two correlating positively with the percentage of SCs. Instead, both control and BrafV600E groups in WT mice displayed a pro-inflammatory and immune stimulatory profile (higher IL17a, IL-5, and IL-12 p70), while SASP-related molecules (CCL-2, IL-6) inversely correlated with the number of recipient B and T cells. RNA-seq performed on donor myeloid and B cells revealed upregulation of inflammatory signaling (IFN-γ, IL-6, and TNF-α) and MHC Class II molecules in both models, implying higher antigen presentation in SCs. Cells collected from WT mice upregulated previously published SC signatures (*SenMayo*, *Sencan*, *Purcell*, *Fridman*, *Pribluda*), while the same cells in NSG mice did not, despite showing other SC markers (*Cdkn2a*). Cells from NSG mice upregulated immune checkpoint genes (*Il10*, *Pdcd1*), while cells from WT mice also upregulated immune response genes (*Cd274*, *Icosl*, *Cd80*, *Cd86*). This data hinted that recipient B and T cells from WT mice efficiently recognized and eliminated SCs, dampening the inflammation. To confirm our hypothesis, we performed the same experiment on Rag1KO mice, lacking B and T cells. Indeed, all Rag1KO mice transplanted with BrafV600E-expressing HSPCs behaved similarly to NSG mice, dying within 40 days post-transplant, showing impaired lymphopoiesis, low levels of immunostimulatory cytokines (IL-5), and no clearance of SCs.

Altogether, we identified two components involved in the clearance or persistence of SCs: the adaptive immunological background of the host and immune checkpoints. In the future, we will test the cited genes and cytokines as potential genotoxicity biomarkers in the context of HSPC GT.

OR013

Results from LIGHT, a first-in-human AAV9-gene replacement therapy trial of HG004, in children and adults with *RPE65*-associated Leber's congenital amaurosis

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Leber's congenital amaurosis (LCA), caused by mutations in the *RPE65* gene (known as LCA2), involves retinal degeneration with nystagmus, night blindness, and severe vision loss in early infancy and childhood. Although an adeno-associated virus serotype 2 (AAV2) gene therapy (voretigene neparvovec-rzyl; Luxturna) has been approved by the FDA and EMA in 2017 and 2018, respectively, AAV2 may not be an ideal vector to transduce the retinal pigment epithelial (RPE) cells than other serotypes such as AAV9. Furthermore, recent real-world data of Luxturna with a high vector dose and large injected volume ($1.5x10^{11}$vg/300uL per eye) suggests foveal thinning and severe progressive chorioretinal atrophy as early as 45 days after the injection (P. Melillo et al., 2024). Here, we developed an AAV9 gene therapy carrying the human *RPE65* gene (HG004) to evaluate its efficacy in *Rpe65*$^{-/-}$ mice and conducted the **LIGHT** (**L**eber's congenital amaurosis **I**nherited blindness of **G**ene t**H**erapy **T**rial), an open-label and dose-escalation study (NCT06088992) to evaluate the safety, tolerability, and efficacy (best-corrected visual acuity, BCVA; full-field stimulus threshold, FST; and kinetic visual field, KVF) of a single subretinal injection of HG004 (AAV9-*hRPE65*) in individuals with LCA2. HG004 demonstrated a significant recovery in scotopic b-wave responses in all treated groups compared to the control group of *Rpe65*$^{-/-}$ mice injected with a vehicle ($p<0.01$). The restoration ratio reached >60% of the wild-type (WT) level and was sustained for 34 weeks. The retinal thickness of HG004-treated and untreated eyes in *Rpe65*$^{-/-}$ mice were 85% and 67% of the WT level, respectively. Based on the preclinical results, 9 adults or children (aged 8-38 years) with *RPE65*-associated LCA have enrolled in the **LIGHT** trial's low, middle, or high-dose cohorts. The average BCVA, FST-white light, and KVF improvements are +8 letters, -2.508 log cd·s/m^2, and +392.75 deg^2 (III4e), respectively at 9-month post-injection ($6×10^9$ vg/200uL per eye), +6.5 letters, -1.408 log cd·s/m^2, and +691.77 deg^2 (III4e), respectively, at 6-month post-injection ($1.0×10^{10}$ vg/200uL per eye), and +15.7 letters, -2.473 log cd·s/m^2, and +763.53 deg^2 (III4e), respectively, at 3-month post-injection ($2.0×10^{10}$vg/200uL per eye). Most subjects, after injection, had at least a 2-log unit increase in pupillary light responses. No serious adverse event or dose-limiting toxicity has been observed. Most adverse events were mild and transient and resolved without intervention. Notably, there were no signs of inflammation, acute retinal toxicity, macular hole, retinal detachment, or severe progressive retinal atrophy after the injection of HG004. These three doses (25-, 15-, and 7.5-fold lower than the approved AAV2-*hRPE65* gene therapy in the Phase 3 trial) in this **LIGHT** trial showed comparable results, suggesting that HG004 may potentially be a safer and more efficacious alternative treatment option for LCA2. HG004 is also being evaluated in a multi-regional clinical trial (NCT05906953) and granted both Orphan Drug Designation and Rare Pediatric Disease Designation by the U.S. FDA.

OR014

AAV HITI for therapy of dominant Retinitis Pigmentosa

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Retinitis Pigmentosa (RP) affects 1 in 3,000 individuals worldwide, with 30-40% of cases having an autosomal dominant (AD) mode of inheritance. The most frequent ADRP (RP4) is due to mutations in *RHO*, the gene encoding for rhodopsin (*RHO*), with several prevalent gain-of-function (GOF) mutations. Conventional gene therapy is ineffective on GOF mutations as removing the mutant *RHO* allele would be required. To this end, we developed an adeno-associated viral vector-mediated homology-independent targeted integration (AAV-HITI) approach directed to *RHO* and optimized the HITI donor DNA design to achieve simultaneous knock-out of the endogenous RHO allele and knock-in of a correct RHO copy. Following AAV-HITI subretinal administration in a humanized mouse model of RP4 we observed that full-length donor DNA integrates predominantly following cleavage from the flanking ITRs, as expected based on its design. Moreover, we show that long-term nuclease expression allows full-length HITI events in the proper orientation. Lastly, subretinal injection of AAV-HITI in RP4 mice results in significant improvement of electrical function, visual acuity and retinal morphology 1-year post-treatment. Our human-centric approach therefore represents a significant advancement towards clinical translation of AAV-HITI for therapy of RP4.

OR015

Suprachoroidal delivery of a vectorized complement inhibitor using a novel AAV capsid as a potential treatment for dry age-related macular degeneration

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Dysregulation of the complement pathway plays an important role in the pathophysiology of the dry form of age-related macular degeneration (dry AMD). Although complement inhibitors are

now approved for treating dry AMD, they require frequent intraocular injections with increased risk of adverse events. We demonstrate that following delivery into the suprachoroidal space (SCS), a novel adeno-associated virus (AAV) vector optimized for ocular expression from this route of administration achieves sustained expression of bioactive complement component 5 (C5) inhibitor in the eye. Multiple formats of purified C5 inhibitors were examined with bio-layer interferometry (BLI) to assess affinities toward human, cynomolgus macaque (NHP), and mouse C5, and all showed sub-nanomolar affinities to their cognate target. C5 inhibitor expression cassettes were packaged in the AAV8 capsid, and their level of ocular protein expression was compared following subretinal injection in mice. A single-chain fragment variable antibody format (anti-hC5-scFv01) demonstrated the highest expression of transgene product in mouse ocular tissues and was selected for further study. AAV delivery of anti-hC5-scFv01 in a mouse sodium iodate outer retinal atrophy model preserved both retinal function and retinal structure. To enhance ocular transduction via SCS administration, we used a directed evolution approach to identify a novel AAV8 variant capsid (7mer peptide insertion at VR-IV) with improved transduction properties. Expression and tolerability of the transgene following suprachoroidal delivery in Yucatan minipigs or in NHPs when expressed either from AAV8 or from the SCS optimized variant were evaluated. Vectors were well tolerated as assessed with *in vivo* imaging. The novel capsid resulted in robust expression of anti-hC5-scFv01 at day 29 postdosing in NHP ocular fluids (n=6, mean: 5 mcg/ml and 25 mcg/ml in aqueous and vitreous humors, respectively) as well as in the NHP macular retina and RPE-choroid (n=5, mean: 15 ng/mg and 84 ng/mg, respectively). *In vivo* expressed anti-hC5-scFv01 present in NHP vitreous humor demonstrated potent inhibition of the target pathway as measured in complement activation assays. In human iPSC-derived RPE cells treated with a source of activated complement, prior transduction with the novel vector expressing anti-hC5-scFv01 resulted in a dose-dependent reduction in formation of terminal complement complex (TCC) on the cell surface. These results support further preclinical development of our AAV-based approach for treating dry AMD, which combines a novel engineered capsid and anti-C5 scFv transgene to achieve sustained complement inhibition in the eye following a single suprachoroidal injection. Our approach offers the potential of a minimally invasive approach to dry AMD treatment that could reduce the treatment burden and deliver therapeutic molecules directly into the site of AMD pathogenesis.

OR016

Preliminary safety and efficacy of DB-OTO gene therapy in pediatric patients with profound deafness due to otoferlin variants: The CHORD phase 1/2 open-label trial

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Otoferlin is critical for inner hair cell (IHC) signal transmission to auditory nerve fibers. Biallelic otoferlin gene (*OTOF*) variants typically cause severe-to-profound deafness. As per preclinical data, DB-OTO gene therapy promotes IHC-selective otoferlin expression from the

human *OTOF* gene, which may instate high-quality hearing. In this first-in-human multicenter, phase 1/2 open-label clinical trial with DB-OTO (CHORD, NCT05788536), safety, tolerability and preliminary efficacy of DB-OTO is evaluated in pediatric patients with profound *OTOF*-related deafness.

DB-OTO is administered by intracochlear injection using a typical facial recess approach through the round window. A 10-month-old female (patient 1), a 4-year-old male (patient 2) and an 18-month-old female (patient 3) received a single intracochlear injection of DB-OTO (7.2 x 10^{12} vector genomes) unilaterally into the inner ear perilymph. A cochlear implant (CI) was placed in the contralateral ear for patients 1 and 2. At baseline, patients 1 and 2 had no detectable hearing by behavioral pure tone audiogram (PTA) or auditory brainstem response (ABR) at maximum tested stimulus levels. Patient 3 responded to very loud sounds (>100 dB).

Through week 24 (patient 1), week 6 (patient 2), and week 2 (patient 3), no dose-limiting toxicities or DB-OTO related adverse events were reported, including absence of vestibular manifestations. Cervical vestibular evoked myogenic potential responses were present at baseline for all 3 patients and remained present post-treatment.

Hearing improvements assessed by PTA and ABR were noted in patients 1 and 2 from the first efficacy assessment (Week 4); patient 3 efficacy assessments have not yet been completed.

At 24 weeks post-treatment, hearing improvement was confirmed in patient 1 with PTA hearing thresholds within normal range at key speech frequencies. Hearing (PTA assessed) was improved by an average of 80 dB across all tested frequencies (baseline thresholds absent at 100 dB, maximum air conduction intensity tested, 250–4000 Hz). These improvements were corroborated by ABR showing thresholds of 45–85 dB through week 24. No improvement from baseline was observed in the untreated ear. Parents and audiologist reported that patient 1 could progressively hear loud then softer sounds when the CI in the contralateral ear was turned off at week 24, consistent with PTA and ABR findings. Global auditory skill development was observed on parent reports and as assessed by the LittlEARS Auditory Questionnaire.

In patient 2, hearing thresholds improved from profound deafness at baseline to severe-to-profound hearing levels at week 6 post-treatment. Hearing improved by an average of 16 dB across all tested frequencies, similar to patient 1 at that timepoint. A present ABR was observed through week 6 (thresholds of 75–90 dB) in the DB-OTO treated ear. Parents reported that patient 2 responded when called by name when the CI in the contralateral ear was turned off.

Updated data for patients 1 and 2, and initial efficacy data for patient 3 will be presented.

These results demonstrated an early overall positive safety and tolerability profile and suggest that DB-OTO gene therapy may significantly improve hearing in patients with profound deafness due to *OTOF* variants.

OR017

Safety and expression of intein-based Dual AAV8.ABCA4 in the non-human primate retina

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We recently demonstrated that split intein-mediated protein trans-splicing can expand AAV

transfer capacity and enable the efficient reconstitution of the large ATP-binding cassette subfamily A member 4 (ABCA4) which is defective in Stargardt disease (STGD1), the most common form of inherited macular degeneration.

Starting from this observation, we showed that subretinal administrations of Dual AAV8.ABCA4 (AAVB-039) improves the phenotype of a STGD1 mouse model.

To further evaluate the translational potential of AAVB-039, a 13-week long non-GLP, ocular safety and expression study was conducted in non-human primates (NHPs). NHPs received a sub-retinal injection of either vehicle (control, CTR) or AAVB-039 at one of two relevant doses, as well as a unique anti-inflammatory regimen.

Ocular inflammation showed a dose-dependent severity, gradually decreasing over time. Anterior inflammation disappeared by week 5, while vitreous cells returned to control levels by week 9 in most eyes.

Electroretinography indicated a mild, dose-dependent reduction in amplitude at week 5, with no abnormalities observed by the study end, except for a borderline reduction in one eye treated at the high dose. Histopathological examination revealed slight to minimal ocular findings, mainly focal and localized to the dosing site, and improving over time. Ocular imaging corroborated these findings.

BaseScope analysis showed nearly total photoreceptor co-expression of mRNAs encoding both ABCA4.intein halves across an extended retinal region, while Simple Western analysis confirmed human ABCA4 protein reconstitution at levels 50% over the endogenous macaque protein.

Our data demonstrate that AAVB-039 can be administered safely to NHPs under an immunosuppressive protocol designed for use in man, and at doses demonstrating robust expression of both mRNA halves and full-length protein. In conjunction with data showing efficacy in animals, this intein-based Dual AAV product shows potential to treat retinal dystrophies, such as Stargardt Disease, using a similar approach.

OR018

PRODYGY: A first-in-human trial of rod-derived cone viability factor (RdCVF) gene therapy in subjects with rod-cone dystrophy

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Rod-cone dystrophy (RCD) is a rare inherited retinal disorder leading to significant vision loss, and for which no treatment is currently available to most patients. SPVN06 is a gene-independent investigational gene therapy expressing the neurotrophic rod-derived cone viability factor (RdCVF) and the thioredoxin RdCVF-Long (RdCVFL), that aims to slow down the progression of central vision loss in patients with RCD, regardless of the underlying pathogenic variant(s).

PRODYGY (NCT05748873) is a first-in-human Phase I//II trial enrolling subjects with advanced RCD due to a mutation in the *RHO*, *PDE6A*, or *PDE6B* gene. The study will assess the safety and

tolerability of a unilateral subretinal injection of SPVN06, one year after treatment administration. Its two-step design includes an open-label dose-escalation phase (Step 1) followed by a controlled, double-masked, randomized, extension phase (Step 2). Step 1 is currently assessing 3 increasing doses of SPVN06, each in 3 subjects with severe advanced RCD. Step 2 will include 3 cohorts of subjects with intermediate advanced RCD. A total of 33 subjects is planned to be enrolled, of whom 27 will receive a single injection of SPVN06 in their worse-seeing eye. Treatment administration was completed in Q2 2023 for Cohort 1 (low dose), and in Q4 2023 for Cohort 2 (medium dose).

Six months after injection in Cohort 1, 7 adverse events (AEs) were reported in treated eyes. In Cohort 2, one month after injection in the last subject, 15 AEs were reported in treated eyes. AEs included but were not limited to macular hole during subretinal injection that resolved without sequelae (n=1), transient mild-to-moderate intraocular pressure elevation, and mild macular edema.

Mild intraocular inflammation was observed in 5 of 6 subjects (2 at the low dose, 3 at the medium dose) and resolved spontaneously within 4 weeks. A transient increase in anti-capsid antibodies, peaking at Week 2, was observed in 2 subjects who had pre-existing antibodies before SPVN06 injection. To date, no dose-limiting toxicities have been observed.

An independent Data Safety Monitoring Board provided a positive recommendation to continue the trial without modifications, and to proceed with treatment administration in Cohort 3 (high dose).

First administration of SPVN06 to patients has shown a favorable safety profile at the low and medium doses so far, with no significant immune response. Treatment of Cohort 3 is currently ongoing.

OR019

CAR-T cells targeting the human IgE-B-cell-receptor specifically abolish IgE production by human B cells and eliminate IgE+ cells *in vivo*

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The prevalence of IgE mediated allergies has risen dramatically in the westernized world. Current treatment for allergies fails to offer a robust cure. In an attempt to eliminate all IgE producing cells, other groupd have designed chimeric antigen receptor (CAR) T cells targeting IgE. However, these CARs target IgE epitopes found both on secreted IgE and on IgE B cell receptors (BCRs), which are membrane bound IgE antibodies. Thus, these previous anti-IgE CAR designs were sensitive to both activation and saturation by free IgE and in some cases also by FcεR bound IgE. To specifically target IgE expressing B cells, Genentech has developed the monoclonal antibody (mAb) Quilizumab. Quilizumab targets an epitope on the M1' domain, found only in the longer of two splice isoforms of IgE BCRs. In adults with inadequately controlled allergic asthma, Quilizumab reduced serum total and allergen-specific IgE by 30–40 %, but had no impact on asthma exacerbations, lung function, or patient-reported symptom measures. We conjecture that

Quilizumab based CAR T cells, will outperform the Quilizumab mAb as well as previous CAR designs, and may serve as a much-needed remedy for IgE mediated allergy.

Quilizumab based 2nd generation CARs ("Q-CAR") were expressed on human primary T lymphocytes via retroviral transduction. We tested the potency of the therapy on engineered NALM-6 cell line expressing membranal IgE (mIgE, modeling a BCR). Q-CAR-T cells successfully eliminated IgE+ expressing NALM6 cells but not wild type NALM6 or NALM6 that express the short isoform of mIgE (lacking the M1` segment). This targeted elimination was accompanied by increase of gamma interferon production by the effector cells. Importantly, the addition of high amounts of free-IgE to the culture did not affect the activity of the CAR-T against its target. In NSG mice, human Q-CAR-T cells controlled expansion of an implanted NALM6-IgE+ leukemia and prolonged survival. To test the potency of the CAR-T cells against primary IgE^+ B cells, which are extremely sparse in blood, we used B cells from human tonsils and induced their class switching to IgE+. Upon 2 days of co culture of isolated B cells with CAR-T cells, or non-transduced T cells as control, our CAR-T cells abolished IgE production, as measured by ELISA, and eliminated IgE secreting cells, as evident by ELISPOT. This elimination was specific to IgE, as IgG production was unaffected. In the near future, we plan to examine the potency of our therapy *in vivo*, in a mouse allergy model. Since mice do not express the M1` domain we designed transgenic mice in which the murine extra membranal proximal domain (EMPD) is replaced by the human one.

Q-CAR-T cells targeting membrane bound IgE are thus envisioned as a curative therapy for IgE mediated allergies, providing sustained surveillance against IgE B cell resurgence and relieving the patient from all atopic diseases.

OR020

Cytosine base editor ameliorates the safety profile of TCR edited T cells for the Adoptive Cell Therapy of gastrointestinal tumors

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Among gastrointestinal tumors, colorectal cancer (CRC) and pancreatic ductal adenocarcinoma (PDAC) are major unmet medical needs, with a high mortality rate mainly related to the onset of metastases, hindering the therapeutic efficacy of conventional treatments. Adoptive Cell Therapy (ACT) with TCR-edited T cells is a promising therapeutic strategy for late-stage patients, but its exploitability is limited by the paucity of TCRs targeting relevant tumor antigens, the presence of an immunosuppressive tumor microenvironment and the safety T cell manufacturing by genome editing tools. To improve the safety profile of T-cell products manufactured by genome editing, we compared a cytosine base editor and CRISPR/Cas9 in their efficacy and safety to generate engineered T cells. We disrupted the endogenous TCR to redirect T cells specificity toward tumor cells, and TIGIT, a major inhibitory molecule in gastrointestinal tumors to enhance T cell ability to counteract immunosuppression. Both editing procedures proved highly efficient in simultaneously disrupting *TRAC*, *TRBC1*, *TRBC2* and *TIGIT* (mean 96.6% and 96.3% of TRAC disruption, 81% and

73.6% of TRBC1 disruption, 75% and 67.3% of TRBC2 disruption and 89.3% and 90.7% of TIGIT disruption with CRISPR/Cas9 and BE4max, respectively) without impairing T cells' memory phenotype differentiation nor their expansion abilities. Remarkably, we only detected translocations between our four target loci in CRISPR/Cas9 treated cells (up to 0.44%). Also, CBE has the potential to act at off-target loci in the genome, mediating sgRNA-dependent and -independent DNA deamination. To assess sgRNA-dependent DNA off-target base editing, we sequenced the top predicted off-target loci for each sgRNA mapping on coding regions. We confirmed that CBE produces high on-target editing with minimal off-target nucleotides conversion. To assess DNA spurious deamination, we performed ultra-deep whole exome sequencing on samples derived from 4 different donors and collected at the end of our manipulation procedure. We observed that CBE efficiently mediated gene disruption without introducing biologically relevant off-target mutations. Then, to select the antigens to target by engineered T cells, we investigated published datasets and identified 19 relevant tumor-associated antigens. By clonal tracking of the TCR repertoire of T cells stimulated with autologous APCs loaded with the selected antigens, we isolated 5 different TCRs specificities. Importantly, these antigens are expressed in our cohort of primary and metastatic PDAC and CRC patients. TCR-edited T cells displayed anti-tumor activity against PDAC cell lines, CRC and PDAC patient-derived organoids (PDOs) while sparing HLA-unrelated and antigen-negative controls. To boost the efficacy of anti-tumor T cells, we coupled TIGIT disruption with TCR editing. In functional assays, $TIGIT_{KO}$ TCR edited T cells engineered with CBE showed superior abilities, compared to TIGIT competent T cells, in eliminating primary and metastatic CRC and PDAC PDOs. In addition, by challenging TCR-edited TIGITKO T cells in an orthotopic model of CRC liver metastases and PDAC primary tumor established from patient-derived organoids, we observed that TIGIT disruption improve the ability of tumor-specific T cells to control tumor growth. Our findings suggest that base editors can generate cellular products with a better safety profile and a promising therapeutic efficacy in gastrointestinal tumors.

OR021

Design of Programmable Immune Reactive Cells (PIRCs) with precision type I Interferon and cytotoxicity response in solid tumors

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Modulating the host immune response is pivotal for achieving successful anti-tumor effects within the tumor microenvironment (TME). Key features, such as immune cell recruitment and activation, cytotoxicity, antigen presentation, and release of type I interferons (IFNs) are all seen as important attributes for mounting a strong and local anti-tumoral response in the TME. Despite recent therapeutic advances, the challenge remains - How can we create cancer immunotherapies that fulfill the requirements of systemic administration while ensuring a precise local response in the TME with minimal systemic immune effects?

Plasmacytoid dendritic cells (pDCs) are a rare lymphoid immune cell that rapidly migrate to sites of inflammation, orchestrating a host immune response against foreign threats. pDCs are renowned for their antiviral functions of eliciting IFNs and inflammatory cytokines. In cancer settings, tumor associated pDCs have been correlated with better outcome, though in some settings they are also found to be immunosuppressed and lack the capacity to mount an IFN

response. In theory, pDCs offer potential in the immunotherapeutic field but face major hurdles by being scarce, fragile, and technically impossible to engineer.

Here, we describe Programmable Immune Reactive Cells (PIRCs) – a first-in-class adoptive cell therapy leveraging pDC-like properties with immune-programmable specificity. Generated from hematopoietic stem and progenitor cells (HSPCs) and combined with the SyNthetic Intramembrane Proteolysis Receptor (SNIPR) system, PIRCs are genetically engineered to prompt an antigen-dependent induction of an immune response, through the stimulation of a STING gain-of-function gene ($STING_{V155M}$).

Preclinically, billions of PIRCs are produced from as little as 100.000 HSPCs under serum-free GMP-compliant conditions. These cells exhibit precision activation towards tumor antigens of choice, triggering a rapid and broad immune response, including type I IFN (mainly IFNα) and chemokines, including numerous CXCR3 ligands. Importantly, PIRCs demonstrate direct killing of cancer cells in both 2D and 3D organoid tumor models, recruitment of immune cells, and elevated NK cell cytotoxic functions. In NSG mice engrafted with solid human tumors, intravenously infused PIRCs infiltrate tumors within 1-2 hours and remain present for days and can induce a local inflammatory signature.

Collectively, PIRC therapy will change the paradigm of current solid tumor therapy by overcoming the immunosuppressive TME. Combining direct killing and immune modulating effects, PIRCs represent a unique precision-programmed effector cell therapy engaging both innate and adaptive immunity. We are currently moving toward a Phase I study to evaluate the safety and efficacy of autologous PIRCs.

OR022

Correlative findings following DSG3-CAART infusion with and without preconditioning in patients with Pemphigus Vulgaris (DesCAARTes™ trial)

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Mucosal-dominant pemphigus vulgaris (mPV) is a painful blistering autoimmune disease mediated by anti-desmoglein 3 autoantibodies (anti-DSG3 Ab). The current standard of care for mPV includes broadly immunosuppressive therapies that have risks of serious or life-threatening infection. We evaluated the safety and activity of a cellular therapy consisting of gene-modified autologous T cells (DSG3-CAART) engineered to specifically eliminate DSG3 reactive B cells in mPV patients (NCT04422912). We have previously reported that mPV patients infused with a single dose of DSG3-CAART cells without preconditioning exhibited linearly increasing CAART cell persistence in circulation at doses ranging from $2x10^7$ transduced cells. Doses exceeding $2.5x10^9$ DSG3-CAART cells (up to $7.5x10^9$ cells) failed to further increase persistence metrics and did not impact serum anti-DSG3 Ab concentrations. We examined additional patient cohorts utilizing two different preconditioning protocols followed by an infusion of $2.5x10^9$ cells as an

attempt to increase total exposure of DSG3-CAART cells. Preconditioning consisted of cyclophosphamide (Cy, n=3, 1000 mg/m^2 over two days) alone or Cy with fludarabine (Cy/Flu, n=3, 1000 mg/m^2 of Cy over two days and 75 mg/m^2 of Flu over three days) along with intravenous immune globulin (IVIG). Here, we report translational data from subjects undergoing these preconditioning regimens with DSG3-CAART cell treatment. Transient leukopenia and lymphopenia were observed in both pre-conditioning arms, with Cy/Flu resulting in a deeper and prolonged lymphocyte reduction than observed with Cy alone. Serum IL-15 concentrations increased modestly in the first week after preconditioning for both Cy and Cy/Flu treated patients. Persistence was unchanged for patients receiving either preconditioning regimen with DSG3-CAART compared to patients receiving DSG3-CAART alone. In a subset of patients, B cell flow cytometric profiling revealed that the impact Cy alone reduced the number of CD19$^+$CD20$^+$ B cells by approximately 90%for 2 weeks following CAART treatment whereas Cy/Flu treatment reduced the number of B cells by approximately 98% within the first 29 days post-CAART treatment. Neither preconditioning regimen fully depleted B cells to undetectable levels at the time points evaluated up to Day 29; in patients who received Cy/Flu B cell repopulation began by Day 29. To date, anti-DSG3 Ab levels remain relatively stable across all dosing and preconditioning cohorts. These data suggest that a single course Cy or Cy/Flu preconditioning regimen alone is unlikely to provide clinical response and that DSG3-CAART has yet to be fully optimized as an effective clinical treatment for mPV patients.

OR023

Restoration of the autophagy machinery promotes long-term persistence and efficacy of CAR-T cells and adoptive cellular products

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Chimeric antigen receptor (CAR)-T cell therapy is a revolutionary approach which produced remarkable clinical responses in patients with B-cell malignancies, however challenges limit their efficacy in other tumours. It has been shown that this limited efficacy is due to the nature of these tumours and the presence of a hostile tumour microenvironment (TME) reducing/abrogating their functionality and persistence.

The aim of the study was to identify metabolic mechanisms responsible for loosing CAR-T and other cellular products' response and develop strategies to improve their efficacy and persistence.

Adoptive cellular products were characterized molecularly (real-time PCR/western blot/RNAseq/proteomics), structurally (electron microscopy (EM)), immunophenotypically (facs/immunofluorescence (IF)), metabolically (seahorse/IF) and functionally (in vitro short- and long-term co-cultures in 2D and 3D models/in a murine model).

Analysing GD2.CAR-T cells from our clinical trial (NCT03373097), we observed that the ex-vivo T-cell manipulation induces a deep and lasting blockage of autophagy pathways independently of the kind of activation, stimulation or cytokines used. We extend our analysis to other cellular

products (antigen-specific cytotoxic T cells and NK cells) observing also a strong autophagy downregulation. Then, we explored whether autophagy can be restored by the over-expression of autophagy key-proteins. This results in an autophagy restoration, increased mitochondrial potential and frequency of naïve cells, better oxygen consumption and extracellular acidification rate. Further, autophagy restored T cells showed a significant reduction in apoptosis, improved response to external stimulus, cell mobility, cell-to-cell communication and intracellular organelle organization. EM analysis also revealed mitochondria colocalization, autophagosomes and micro-vesicles. Omics data showed the activation of both adaptive and innate immune system as well as upregulation of antigen presentation and immunological synapse formation and a positive response to stress conditions. At the functional level, autophagy-restored CAR-T cells eradicate more efficiently tumour at low effector:target ratios in both short and long-term co-cultures using 2D and 3D models as well as in a murine model without signs of exhaustion or toxicity.

In conclusion, autophagy restoration in CAR-T cells and adoptive cellular products could revolutionize the effectiveness of these approaches securing better persistence, anti-tumour activity and ability to resist to negative stimuli induced by the tumour and its TME.

OR024

Base-edited CAR38 T cells evade fratricide and immune rejection while delivering potent anti-leukemic affects

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Despite improved treatment options, relapsed/refractory hematologic malignancies often have a poor prognosis, even after allogeneic stem cell transplantation. Cluster of differentiation 38 (CD38) is a single-chain cell surface transmembrane protein with nicotinamide adenine dinucleotide glycohydrolase (NADase) activity, which modulates intracellular NAD^+ and contributes to T cell activation, differentiation, and bioenergetic state. CD38 exhibits limited expression on non-hematopoietic tissues and is under investigation as an immunotherapy target for hematologic malignancies. There is extensive clinical experience using anti-CD38 monoclonal antibodies, and early trial data from autologous anti-CD38 chimeric antigen receptor (CAR) T-cells provide promising safety and efficacy profiles.

We describe the development of ‘universal’ allogeneic CAR38 T-cells using a combination of lentiviral delivery and base editing knockouts. Initially, the expression of CAR38 (comprising a Daratumumab-derived single-chain variable fragment (scFv)) resulted in ‘self-lysis’ of activated T-cells and poor cell yields. Pre-emptive knockout of CD38 on T-cells by cytidine base editing resolved fratricide issues with >70% knockout, rising to 100% by self-enrichment in CAR38 transduced cells. Appropriate C>T conversions were verified by targeted sequencing. Importantly, CD38 knockout did not impact *in vitro* cytotoxic function when tested in either CAR38 T-cells or control CAR19 T-cells. Additionally, there was no apparent reduction in metabolic fitness after CD38 knockout when assessed by Agilent Seahorse XF analyzer testing spare respiratory capacity.

For ‘universal’ donor iterations, multiplexed base editing of the *T Cell Receptor Beta Constant 1/2* (*TRBC1/2*), *Beta-2-Microglobulin* (*β2m*), and *Regulatory Factor X5* (*RFX5*) loci was incorporated to mitigate against graft-versus-host disease (GVHD) and protect against host-mediated immune rejection of human leukocyte antigen (HLA) mismatched cells. Flow cross-match assays in the presence of human serum with defined anti-HLA antibodies demonstrated evasion of pre-existing humoral immunity when both HLA class I and II were removed. Similarly, mixed lymphocyte cultures and proliferation assays suggested that allogeneic T cell-mediated responses against HLA-mismatched CAR T-cells were eliminated by disruption of both HLA class I and II expression. In potency experiments, ‘universal’ CAR T-cells were able to inhibit $CD19^{+}CD38^{+}$ Daudi B cell malignancy in humanized NOD/SCID/gamma (NSG) immunodeficient mice. When leukemic progression was tracked by IVIS imaging over 7 weeks, untreated animals reached termination thresholds within 31 days, whereas CAR19 groups survived to 40 days, and CAR38 animals survived the longest, to 53 days ($p<0.005$). These results support further investigation and development of ‘universal’ CAR38 T cell strategies for relapsed/refractory blood malignancies, including acute lymphoblastic leukaemia, acute myeloid leukaemia, and myeloma.

OR025

Efficient Retinal Transduction and Excellent Inhibitory Effect on Retinal Detachment of EXG202 delivered by intravitreal injection in NHP and a Severe Neovascularization and vascular leakage Mouse Model

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The success of rAAV-based gene therapy for most ocular diseases depends on efficient delivery of the vector to the photoreceptors (PR) and/or retinal pigment epithelium (RPE). While subretinal administration provides the most direct access to the PR and RPE, it is more invasive, with a risk of damage to the retinal tissue. Intravitreal (IVT) injection is a safe, non-surgical procedure and have been widely used for delivery of protein therapeutics to treat common retinal diseases.

Here, we report the validation of EXG202, a novel AAV-based gene therapeutic vector expressing a fusion protein that binds all subtypes of VEGF as well as ANG2 and packaged in a novel engineered AAV capsid. The test systems are NHP and the Tet/opsin/VEGF double transgenic mouse model. In NHP, a total of 6 animals were administrated with EXG202 intravitreally in one eye and AV7m8 (the benchmark IVT control vector) in the contralateral eye (1×10^{11}vg/eye). AV7m8 is a rAAV vector containing the same transgene expression cassette as that of EXG202 but packaged in AV7m8 capsid. Post injection, animals were followed for 8 wks. Levels of the transgene product in aqueous humor (AH) and vitreous humor (VH) collected at Week 4 & 8, as well as the ocular tissues (iris-cilia body, retina, RPE/choroid) collected at wk 8 were determined. The overall transgene product levels in eyes received EXG202 is up to 3-fold higher than that in eyes received AV7m8.

The superiority of retinal transduction of EXG202 delivered by intravitreal injection was further demonstrated in a dose-range finding study in the Tet/opsin/VEGF double transgenic mice, a severe neovascularization and vascular leakage mouse model. The phenotype and mechanism behind this model are highly relevant to nAMD. Adult Tet/opsin/VEGF mice (n = 8-10/group) were given an intravitreal injection of EXG202 of 1×10^{8}, 3×10^{8}, and 1×10^{9} vg/eye, or the negative

control vector AVT-Luc in the other eye. Four weeks post injection, the mice were given doxycycline to turn on expression of VEGF in photoreceptors. Four days later, mice were anesthetized, fundus photographs obtained and examined by a masked investigator and were determined to show no, partial, or total exudative retinal detachment. Reduction of total retinal detachment by 75% to 100% was observed in the Tet/opsin/VEGF double transgenic mice with 1×10^{9}, 3×10^{8}, and 1×10^{8} vg of EXG202, demonstrating a robust rescue effect on the retina detachment caused by severe neovascularization and vascular leakage of the mouse model.

In conclusion, EXG202 demonstrated a superior retinal transduction efficiency to the benchmark control vector AV7m8 in NHP delivered by intravitreal injection. More importantly, reduction of total retinal detachment by 75% to 100% was observed in the Tet/opsin/VEGF double transgenic mice with doses of 1×10^{8}, 3×10^{8}, and1×10^{9} vg of EXG202. Taken together, data from this study strongly support clinical development of EXG202 in patients with nAMD or DME.

OR026

OTOV101 Gene therapy for autosomal recessive deafness 9: a multicenter, open-label, single-arm, investigator initiated intervention study

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Autosomal recessive deafness 9 (DFNB9) is a congenital auditory neuropathy with clinical features including congenital or prelingual, bilateral symmetry, severe to complete deafness, caused by OTOF mutations. We previously reported the safety and efficacy of the gene therapy of adeno-associated virus (AAV) mediated OTOF delivery (OTOV101N + OTOV101C Injection) in two children (5- and 8-year-olds) for the first time worldwide (Qi J, et al. Adv Sci (Weinh). 2024;11(11): e2306788). The treatments can restore hearing in children with DFNB9. We continued to enroll more subjects including the toddler, adolescent and adult, and more subjects who needed bilateral treatments.

This study is a multicenter, open-label, single-arm investigator-initiated intervention trial. We recruited 9 DFNB9 patients with age diversity (1.5 to 23.9-year-old) at 4 China sites for treatment with the OTOV101 at a dose of 8.4*10^11 to 11.2*10^11 vg per ear. The follow-up period will be 5 years post treatment. The primary outcomes are safety and tolerability. Secondary outcomes include auditory function assessments.

We present a relevant evaluation of safety and efficacy in 9 patients for 1to 9 months after OTOV101 treatment. 2 subjects were toddlers (1.5 and 1.9-year-old), and 1 subject was adult (23.9-year-old). 2 subjects received bilateral treatments. No serious adverse events (SAE), nor AE led to discontinuation, nor death occurred. A total of 8 treatment- emerged adverse events (TEAE) which graded as I and II were observed. Hearing function improved in 11 injecting ears of 9 patients from 1 month after surgery. The mean Click Auditory Brainstem Response (ABR) of 1 month after surgery decreased from >99 dB at baseline to 56.7dB. Among them, the thresholds of Click-ABR, tone-burst ABR (TB-ABR) and pure tone audiology (PTA) in the 23.9-year-old adult patient decreased from >100 dB, >100 dB, and 93.6 dB at baseline to 70, 81, 67.9 dB at 1 month. The hearing recovery of patients showed a potential age correlation. Patients with hearing recovery were divided into three groups by age (1: 1-3 years; 2: 3-12 years; 3: >14 years in order).

The thresholds of Click-ABR (PTA) in the 3 groups were improved by 45 dB (7.9 dB), 51.8 dB (56.7 dB) and 20 dB (25.7 dB) from baseline at 1 month, respectively. Overall, patients aged 3-12 years had a better hearing recovery.

In this trial, OTOV101 gene therapy had been proved as a safe and effective treatment for infants to young adult DFNB9 patients, which appears to be age-related therapeutics and indicated a better treatment window (age 3-12 years old) of AAV mediated gene therapy for DFNB9. The trial has been registered with ClinicalTrials.gov, NCT 05901480, and is still ongoing.

OR027

GNT0004, Genethon's AAV8 vector-delivered microdystrophin gene therapy for Duchenne muscular dystrophy: first data from Phase 1/2 part of GNT-016-MDYF all-in-one clinical trial in ambulant boys

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Duchenne Muscular Dystrophy (DMD) is a rare, progressive, lethal, X-linked disease caused by mutations in the dystrophin gene, leading to progressive muscle degeneration and early death.

GNT0004 is a recombinant serotype 8 adeno-associated virus (AAV8) vector-based gene therapy containing a shortened functional version of dystrophin gene (hMD1). Driven by the Spc5.12 promoter, hMD1 transgene targets skeletal and cardiac muscles.

The pharmacodynamic (PD), safety/tolerability, and efficacy of a single IV administration of GNT0004 is evaluated in the clinical trial GNT-016-MDYF. This all-in-one phase 1/2/3 international trial combines: a first-in-human dose escalation phase (Part 1), a quadruple blind, placebo controlled, pivotal Phase 3, with a 1:1 randomisation (Part 2), at the end of Part 2, there is a cross-over administration of GNT0004 and a long-term follow-up (Part 3). The planned sample size of 64 patients will be reassessed at the interim analysis.

Ambulant DMD boys aged 6 to under 10 years, with a stable or early declining North Star Ambulatory Assessment (NSAA) score despite stable steroids treatment, and no neutralising antibodies to AAV8 were included. Eligible participants were rolled over from the natural history / baseline study (GNT-014-MDYF) into GNT-016-MDYF. Participants received immune prophylaxis with sirolimus and add-on steroids. hMD1 expression was measured by immunohistochemistry and Simple Western at baseline and 8 weeks after GNT0004 administration (biceps brachii biopsies).

Here we report data from Part 1. Two and three participants received dose 1 (1x1013vg/kg) and dose 2 (3x1013vg/kg) respectively (follow-up per patient ranged from 26 weeks to 3 years). Post dosing, mean hMD1 positive fibres was 1.96% with dose 1, and 53% with Dose 2. Vector Copy Number per nuclei (VCN) was 0.4-2.5 (mean 1.2) with Dose 2. A decrease in serum creatine kinase (CK) of 50%- 87% from baseline to week 16 (a timepoint after cessation of immune

prophylaxis), with a sustained decrease observed with dose 2. Administration of GNT0004 was safe and well tolerated in all subjects receiving sirolimus and steroid prophylaxis, which was initiated after dosing of patient 1 in cohort 1 (SUSAR case). Five Adverse Drug Reactions were reported in the two cohorts, including one serious adverse reaction (SUSAR) of immune-mediated myositis that occurred in the first patient in cohort 1 (dystrophin epitope-naive patient at risk of immunological complications, this subpopulation was consequently excluded from the trial) and 4 mild adverse events (occurring in 3 patients).

GNT0004 at dose 2 provided significant transgene transduction and expression in skeletal muscle. hMD1 expression appeared to be correctly localised to the sarcolemma, which may contribute to stabilisation of the dystrophin-associated glycoprotein complex. The early and sustained decrease in CK suggests sarcolemma stabilisation with preliminary evidence of clinical benefit. GNT0004 administration was safe and well tolerated in the last 4 participants (including all 3 patients at dose 2). Therefore, dose 2 (3x1013vg/kg) was selected to proceed to part 2 (pivotal phase 3).

OR028

Results from GALILEO-1, a first-in-human clinical trial of FLT201 gene therapy in patients with Gaucher disease Type 1

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FLT201 is an investigational AAV gene therapy for the treatment of Gaucher disease Type 1 (GD1). FLT201 contains a unique *GBA1-85* transgene that encodes an engineered variant of beta-glucocerebrosidase (GCase85) under control of a liver-specific promoter. GCase85 has two amino acid substitutions that increase its stability by approximately 6-fold in serum and 20-fold at lysosomal pH compared to wild-type GCase. FLT201 uses a proprietary capsid (AAVS3) constructed by rational design that efficiently transduces human hepatocytes and enables high expression with low vector doses. A one-time infusion of FLT201 has the potential to lead to durable endogenous expression of a highly stable form of GCase, thereby eliminating the need for chronic treatment with enzyme replacement therapy (ERT) or Substrate Reduction Therapy (SRT). The increased stability of GCase85 may also increase its tissue coverage compared to ERT and SRT.

GALILEO-1 is a first-in-human, dose-finding study of a single IV infusion of FLT201. Eligible patients have GD1, are aged 18 years or older, are receiving an approved GD1 therapy and have a negative AAVS3 neutralizing antibody test. Study objectives are to assess the safety and tolerability of FLT201 and to investigate the relationship of FLT201 dose to GCase85 expression with plasma substrate concentration and clinical parameters. The starting dose of FLT201 for the first cohort is 4.5 x 10^{11} vector genomes per kilogram bodyweight (vg/kg) with subsequent dose escalation, if required, based on observed safety and efficacy. Participants will be followed for 38 weeks after treatment before entering long-term follow-up.

6 patients (4 males and 2 females) have been enrolled into the trial and have received FLT201 at the same dose of 4.5 x 10^{11} vg/kg. Duration of follow-up at the time of this abstract submission ranges from 14 weeks to 11 months. All patients tolerated the infusion well with no infusion related reactions reported. Previously presented data [ASGCT – Baltimore May 2024]: Safety: no

serious adverse events or dose-limiting toxicities. Mild-moderate increases in ALT have been managed with immune suppression medication with no impact on efficacy. Efficacy: the first 4 patients, who had discontinued their Gaucher treatment, have shown continuous, durable GCase expression from the *GBA1-85* transgene with maintenance or improvement of hemoglobin and platelets and stable liver and spleen volumes. A marked reduction in lyso-Gb1 has been observed in patients with elevated lyso-Gb1 at baseline, before and after discontinuation of usual ERT/SRT treatments. Bone marrow burden, measured by MRI, has improved in all 4 patients. Additional safety and efficacy data from all 6 treated patients will be presented.

OR029

Enhanced Immunotherapy for High-Grade Gliomas Using Oncolytic Virus Armed with Co-Stimulatory Molecule and EphA2-Engager

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Pediatric high-grade gliomas (pHGGs) are among the most common malignant neoplasms of childhood: their outcome remains dismal and patients suffer from invalidating long-term sequelae.

To enhance the suboptimal antitumor efficacy of oncolytic adenoviruses (OAs), we tested an innovative gene-therapy approach based on an OA armed with the CD28 T-cell co-stimulator ligand CD86 (OA86) and a bispecific T-cell engager (BiTE) targeting the erythropoietin-producing human hepatocellular carcinoma A2 receptor (EphA2), delivered by a replication-incompetent adenoviral vector (EAd).

The antitumoral effect of the combinatorial approach was evaluated *in vitro* by infecting two HGG lines (U373/U87) with OA86 alone, EAd alone, or both viruses. T cells, isolated from healthy donors, were co-cultured with infected tumor cells at 10:1 ratio. In both lines, the presence of BiTE demonstrated significant anti-tumor efficacy (% apoptotic cells, EAd+PanT vs NT+PanT: 88.17±6.92 vs 20.50±25.63, p=0.0022 in U373; Ead+PanT vs NT+PanT: 38.82±18.29 vs 8.98±4.84, p=0.0032 in U87); in the more resistant U87 cell line, OA86+EAd treatment in combination with T cells significantly increased tumor control (% apoptotic cells, OA86+EAd+PanT vs EAd+PanT: 79.03±10.40 vs 38.82±18.29, p=0.0009). To elucidate the role of the T-cell response, we analyzed the phenotype of T cells at the end of the co-culture and observed a significantly increased expression of activation and proliferation markers CD25, CD95 and Ki67 ($p<0.05$) in both CD4+ and CD8+ T cells in the presence of EAd, as compared to OA86. This effect is further enhanced when the more resistant U87 cell line is infected with OA86+EAd.

Since T regulatory cells (Tregs) are known for their immunomodulatory capacities, their infiltration in HGGs and their induction through the CD28-pathway, an integrative evaluation was conducted to understand their role in the approach. In short-term (4 days) co-cultures with infected U373/U87 cells, expanded Tregs did not show antitumor activity in the presence of BiTE (% apoptotic cells, EAd+Tregs vs NT+Tregs: 8.62±5.64 vs 5.65±1.05, p=0.234 in U87). Suppression assays, performed to assess the suppressive capabilities of the expanded Tregs on conventional CD4+ and CD8+ T cells, confirmed their immunomodulatory potency: CD4+ and CD8+ T cells co-

cultured with Tregs exhibited marked inhibition of proliferation in a dose-dependent manner as compared to CD4+ and CD8+ cultured alone ($p<0.05$).

The combinatorial approach was also tested *in vivo* in 2 different murine models. First, following the orthotopic engraftment of HGG cells, intratumoral infusion of OA86+EAd and human FFLuc-T cells resulted in enhanced persistence of T cells in the brain, as assessed by bioluminescence (treated vs NT: 1.623±0.341 vs 0.836±0.058, $p=0.0020$ at day 29 post-infection). Then, in a humanized mouse model developed via sublethal irradiation and subsequent infusion of human CD34+, we observed a prolonged tumor control over time in the OA86+EAd group compared to the NT, with a 3.25-fold decrease of the tumor bioluminescence 80 days post-viral infection.

In conclusion, combining oncolytic virus activity and T-cell activation via OA86 and EphA2-BiTE gene therapy successfully induces long-lasting tumor control in several HGG models, including a humanized model. This strategy could represent a more effective and less toxic therapeutic alternative than conventional treatments.

OR030

A cancer immunotherapy modality based on dendritic cell reprogramming *in vivo*

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Dysfunctional antigen presentation mediated by MHC downregulation and low infiltration of functional professional antigen presenting cells (APCs) are critical immune evasion mechanisms underlying intrinsic resistance to immunotherapies. Among APCs, conventional type 1 dendritic cells (cDC1s) in tumors are key drivers of efficient anti-tumor immunity and associated with better patient prognosis. We have recently demonstrated that overexpression of transcription factors PU.1, IRF8 and BATF3 (PIB) drives cell fate reprogramming of tumor cells into functional antigen-presenting cDC1s, which upon *in vivo* transfer mount efficient anti-tumor immunity in immune checkpoint blockade (ICB)-resistant models. To circumvent *ex vivo* cell manipulation challenges, Asgard Therapeutics is developing AT-108, a novel immunotherapy based on *de novo* recreation of cDC1s´ functional properties in tumor cells by *in vivo* direct reprogramming, to restore tumor immunogenicity and overcome immune evasion.

Here, we developed an approach to reprogram tumor cells *in vivo* by adenoviral vector (Ad) delivery of PIB, which enabled them to present endogenous antigens as cDC1s. We started by exploring *in vivo* cDC1 reprogramming and anti-tumor efficacy upon subcutaneous implantation

of *ex-vivo* transduced cancer cells in ICB-resistant syngeneic and xenograft models. *In vivo* induced cDC1s drove tumor regression in YUMM1.7 (100% complete response, CR) and $BRAF^{V600E}$ $COX2^{KO}$ (50% CR in cDC1-deficient mice) models and synergized with ICBs leading to tumor regression in B16 model (40% CR), expansion of tumor-reactive T-cells and abscopal effect, indicating that reprogramming reverses MHC downregulation and replenishes cDC1s in tumors promoting anti-tumor immunity. Interestingly, reprogrammed tumor cells remodeled their tumor microenvironment by recruiting lymphocytes and promoting the formation of tertiary lymphoid-like structures and expansion of polyclonal cytotoxic T cells. PIB-transduced human cancer cells acquired cDC1 (CLEC9A, XCR1) and antigen-presentation markers (MHC-I/II, CD40/80) *in vivo* with similar efficiency and kinetics as *in vitro*, and the cDC1 reprogramming process was not hindered by the presence of immunosuppressive cells or cytokines. To select an optimal platform to express PIB within tumors, we compared transduction and reprogramming capacity of PIB-encoding replication-deficient Ad and adeno-associated viral (AAV) to lentiviral (LV) vectors. We observed that in contrast to AAV-PIB, Ad-PIB induced cDC1 phenotype with similar efficiency as LV-PIB in patient-derived cancer spheroids and promoted infiltration and activation of HLA-A2 – matched CD8+ T cells. Additionally, Ad allowed high *in vivo* transduction of tumors, supporting Ad as preferred vectors for AT-108. Remarkably, intra-tumoral injection of AT-108 allowed *in situ* phenotypic reprogramming and enhanced infiltration of cytotoxic T cells, drove complete tumor regression in B16 model (50% CR) and induced abscopal effect. Finally, CR mice treated with Ad-PIB mounted long-term immune memory, remained tumor-free for 160 days and were protected against local and metastatic colonization after subcutaneous and intravenous re-challenges.

Together, our study paves the way for the first-in-human trials and provide preclinical proof-of-concept for AT-108 as an off-the-shelf, personalized cancer immunotherapy based on *in situ* cDC1 reprogramming.

OR031

An hemato-chimeric mouse model hosting human liver-resident macrophages for translational studies using *in vivo* lentiviral-based gene therapies

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Liver metastases are associated with poor response to current pharmacological treatments, including immunotherapy. We have previously described an in vivo gene therapy platform, based on lentiviral vectors (LVs) to selectively engineer liver macrophages to secrete type I interferon (IFNα) within liver metastases. Gene-based IFNα delivery caused reduced metastatic volume of both colorectal and pancreatic ductal adenocarcinoma liver metastases in mice. Therapeutic response was associated with macrophage immune activation, increased antigen presentation and increased CD8 T cell infiltration and cytotoxicity. In a subset of mice, we detected resistance to IFNα therapy, associated with increased IL-10 signalling, expansion of Eomes CD4+ T cells, a cell type displaying features of type I regulatory T (Tr1) cells, and CTLA-4. Combination of in vivo IFNα gene therapy with CTLA-4 immune checkpoint blockade expanded tumor-reactive T cells, attaining complete response in most mice. These findings support a promising therapeutic

strategy with feasible translation to patients, however human-specific myeloid restriction to LV transduction poses a challenge. Among other mechanisms, SAMHD1-mediated interference with LV retrotranscription and integration in the human genome limits the potentiality of our platform. Here, we introduce a novel mouse model featuring human KCs, alongside LVs customized for human macrophage-specific transgene expression. We established the presence of human KCs in mice by administering in vivo LVs expressing human myeloid-specific growth factors, followed by transplantation of human hematopoietic stem cells. Compared at single cell-RNA level, human KCs predominated in the livers of hemato-chimeric mice and exhibited transcriptional profiles akin to patient-derived KCs. Additionally, we developed and characterized a human macrophage specific MRC1 promoter. Notably, expression driven by the human MRC1 promoter yielded significant transgene output, with heightened expression observed in M2-polarized macrophages. The development of this humanized mouse model represents a pivotal advancement for LV-based in vivo gene therapies. Humanised models not only enhance our understanding of LV-mediated transduction in human macrophages but also accelerate the translation of preclinical findings to clinical applications.

OR032

Genetically modified bacteria as anti-cancer Trojan horse; intratumoural delivery of immunotherapy by *Clostridium sporogenes*

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In recent years, cancer immunotherapy has revolutionized; there is an ongoing growth in novel immunotherapeutics to treat various cancer types. Although remarkable successes, ineffective treatment or adverse effects in a subset of patients due to systemic exposure remain major challenges. Precise delivery methods are investigated to tackle these limitations and increase therapeutic efficacy. Most solid cancers contain areas of hypoxia and necrosis, which are associated with poor prognosis. Strikingly, such necrotic environment is specific for neoplasia and therefore provides an opportunity for tumour-specific treatment. We study the use of the anaerobic bacteria *Clostridium sporogenes* as targeted therapy. Upon spore injection, this species selectively penetrates necrotic tumour areas and germinates into vegetative bacteria. We have developed innovative genetic tools to “arm” *C. sporogenes* with a variety of immunotherapeutic genes. These novel strains can act as an intratumoural immunotherapeutic producing and secreting vehicle, a Trojan horse strategy. The most efficient genome editing strategy has been optimized and then used to genetically modify *C. sporogenes*, whereby immunotherapeutic genes murine interleukin (mIL)-2, murine granulocyte-macrophage colony-stimulating factor (mGM-CSF), and anti-programmed death ligand 1 (aPD-L1) were inserted into the chromosome of this bacteria. Immunotherapeutic production/secretion and biological activity were assessed using ELISA and cellular assays, respectively. Additionally, a 3D cellular model was investigated for mimicking tumour necrosis and to function as *in vitro* validation strategy to study bacterial colonization specific aspects. Our results showed that the CRISPR-Cas12 technique was most efficient for clostridial genome editing and sequencing results confirmed insertion of therapeutic genes into the chromosome of *C. sporogenes*. To allow *in vitro* validation in cell models, two different spheroid models have been optimized: Lewis Lung Carcinoma spheroids and CT26 spheroids. Both models were able to mimic tumour necrosis whereby LLC spheroids developed a volume and necrotic fraction approximately two-fold larger compared to CT26 spheroids. When

spores were added, colonization was detected for both models within 24 hours post-administration, with colonization levels up to $2.5x10^5$ and $1,75x10^6$ colony forming units (CFU)/spheroid for LLC and CT26, respectively. Using these spheroid models, we verified that the genetic insertions did not affect the sporulation, growth, or colonization abilities of the genetically modified bacteria. Additionally, ELISA results showed successful secretion of mIL-2 and mGM-CSF up to 3400 and 240 pg/mL (respectively) from both overnight cultured bacteria and intra-spheroid colonization. Our results show our successful development of immunotherapeutic producing *Clostridium* strains with the potential to secrete immunotherapy specifically in necrotic tumour area.

OR033

Mycobacterium Tuberculosis polyprotein mRNA vaccines designed to augment protein sorting enhance immunogenicity and protection in preclinical studies

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Pulmonary Tuberculosis (TB) is a contagious infectious disease caused by the bacterial pathogen Mycobacterium tuberculosis (M.tb). Infection is associated with high rates of morbidity and mortality, particularly in middle and low-income countries. Despite implementing neonatal BCG vaccination programmes, South Africa continues to have one of the highest TB incidence rates in the world. While BCG vaccines confer protection against TB in children, age-related waning of the immune response leaves adolescents and adults vulnerable to infection. Complementary vaccination strategies aimed at preventing infection or controlling disease progression would help reduce disease burdens in endemic countries. To this end, we designed next-generation mRNA vaccine candidates based on four priority antigens, identified from T cell receptor profiling of individuals who were able to control M.tb infection versus those that progressed to tuberculosis.

Twenty three M.tb mRNA vaccine candidates were designed to express CFP-10, WbbL1, PE-13 and PPE-18, either as individual antigens or as polyproteins, with or without complementary leader sequences to facilitate intracellular transport and protein sorting. Sequences were modified to improve in situ translation and cloned into an mRNA plasmid template. Synthetic mRNAs were synthesised by in vitro transcription using modified nucleotides and co-transcriptionally capped with CleanCap® reagent AG. Size and integrity of mRNA transcripts was confirmed by capillary gel electrophoresis (CGE) while purity and concentration were confirmed using spectrophotometry. Microfluidics was used to formulate the mRNA as a four-component ionisable lipid nanoparticle (LNP). Particle size and polydispersity was measured using dynamic light scattering, and encapsulation efficiency using the Quant-it™ RiboGreen RNA Assay.

Preclinical immunogenicity studies were performed in BALB/c, C57BL/6, and C3HeB/FeJ (Kramnik) mice. Three different strains were selected as MHC haplotype affects T cell recognition of M.tb antigens. Mice were vaccinated intramuscularly with M.tb mRNA vaccines or a control (LNP-mFLuc), with a boost three weeks post prime. Spleens were harvested for ex-vivo T cell stimulations using peptide pools specific to each of the four proteins. Secreted cytokine profiles (increased IFN-γ, TNF-α, IL-2) indicate that vaccination resulted in an M.tb antigen-specific Th-1

immune response. Furthermore, intracellular cytokine staining showed an increase in the percentage of IFN-γ positive CD4 T cells (CD3+CD4+IFN-γ+). Interestingly, leader sequence selection altered the immunogenicity profile of single and polyprotein vaccine candidates. Furthermore, the arrangement of the antigens within the polyprotein impacted T cell activation.

Selected mRNA vaccine candidates were further evaluated in murine challenge studies. Vaccines that elicited strong antigen-specific T cell responses, but differed in transcript design, leader sequence, or polyprotein arrangement were chosen. C3HeB/FeJ (Kramnik) mice were vaccinated with mRNA candidates or BCG Danish 1331, and received low-dose aerosol challenge with M.tb nine weeks later. Bacterial burdens were measured in the lungs, where BCG and two mRNA vaccine candidates showed a significant reduction in CFU's when compared to unvaccinated mice, suggesting immunization was achieved. Analysis of T cell subsets and cytokine profiles is currently underway. Two priority vaccine candidates have been selected to advance to Phase 1 clinical trials and are currently in the product development pipeline.

OR034

Novel adenovirus vaccine vectors lacking binding to the thrombosis associated Platelet Factor 4 protein

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The adenoviral (Ad) vector based AstraZeneca and Janssen COVID vaccines have been linked to rare cases of vaccine-induced thrombotic thrombocytopenia (VITT), a condition that appears to be linked to Ad binding to the blood protein Platelet Factor 4 (PF4). Meanwhile, several adeno-associated virus vectors (AAVs) in clinical use for gene therapy have been associated with cases of thrombotic microangiopathy (TMA), although a potential link to PF4 binding remains yet to be identified.

Harnessing the broad diversity of natural and synthetic Ads and AAVs, we strived to identify vectors that do not interact with PF4, to locate the binding site of PF4 on commonly used Ad vectors and to establish vaccine vector platforms using these PF4-non-binding Ad vectors.

Using a range of techniques, including SPR and ELISA-qPCR, we assessed the PF4 binding ability of 12 AAV and 50 Ad vectors including genetically or chemically modified Ad5 hexon or fiber variants. Unlike most of the vectors tested, AAV1, AAV6 and AAV7, as well as Ad34 and Ad80 did not bind to PF4. Likewise, the deletion of the hexon hyper-variable region (HVR) 1 of Ad5 or its shielding by PEGylation ablated the vector's PF4 binding. This suggests that HVR1 is the primary binding site of PF4 on the Ad5 capsid, which was supported by Brownian Dynamics (BD) modeling of molecular interactions. These simulations indicated further that PF4 binding is primarily driven by HVR1 loop steric occupancy of the hexon surface rather than by its electrostatic potential, suggesting that genetic manipulations that modify HVR1 surface charge are unlikely to ablate Ad binding to PF4 unless the loop is also shortened.

We then constructed PF4 non-binding Ad vaccines expressing the SARS-CoV-2 spike S1 domain and tested their immunogenicity in mice. The candidate COVID vaccine vector derived from HVR1-deleted Ad5 induced robust S1-specific CD8 and CD4 T cell responses matching those elicited by the parental Ad5 vector. Interestingly HVR1-deleted Ad5 did not induce detectable vector-binding antibody titers, suggesting an improved potential for homologous prime-boost vaccination. The Ad34-based vaccine vector also induced anti-spike antibody responses, although at lower levels than those elicited by the parental Ad5 vector.

Taken together, we document important advantages of novel PF4-non-binding adenovirus vectors for vaccine delivery, raising expectations for safer vector platforms with decreased risk of VITT in prophylactic as well as therapeutic applications.

OR035

Tailoring the adjuvanticity of lipid nanoparticles by PEG lipid ratio and phospholipid modifications

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Lipid nanoparticles (LNPs) represent the leading delivery platform for mRNA vaccines with advantageous biocompatibility, scalability, adjuvant activity, and often an acceptable safety profile. We investigated the physicochemical characteristics and adjuvanticity of four-component LNPs. Previous vaccine studies have demonstrated that altering the ionizable lipid influences the adjuvanticity of an LNP; however, the impact of the polyethylene glycol (PEG) lipid and phospholipid has received less attention. Our mRNA-LNP vaccine formulations utilized different phospholipids and varying ratios of PEG lipid, while the ionizable lipid and cholesterol remained approximately constant. We demonstrate that such modifications impact the magnitude and quality of the vaccine-elicited immune responses. A pivotal finding of our study is that mRNA-LNP vaccines, when designed with neutral zwitterionic phospholipids such as distearoyl-sn-glycero-3-phosphocholine (DSPC) and a PEG lipid molar ratio of 0.5%, result in higher binding antibody production with improved virus neutralization capabilities and a more robust memory B cell and long-lived plasma cell response in mice. These enhancements can be attributed to an increase in protein synthesis within key immune sites such as the spleen, draining lymph nodes, and the vaccination site, accompanied by heightened levels of IL-6 in the draining lymph nodes, more efficient uptake by dendritic cells, and macrophages, and improved ability to release endocytosed mRNA into the cytoplasm from endosomes. On the other hand, an LNP that contains negatively charged phospholipid 1,2-dioctadecenoyl-sn-glycero-3-phosphoserine (DOPS) has been found to induce strong $CD8^+$ T cell responses in both infectious disease and cancer vaccine settings. Taken together, these findings support the rational design of novel LNPs to tailor immune responses (cellular- or humoral-focused) based on the vaccine application.

OR036

GRAd as vaccine platform for COVID-19, HIV, and global health

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The GRAd platform, based on a low-seroprevalent gorilla-derived adenoviral vector, represents a transformative approach for the rapid generation of vaccines against a variety of pathogens, proving especially suitable for outbreaks and experimental medicine. ReiThera's pioneering efforts in the development of simian adenoviral (SAd) vector vaccines have led to the isolation of several SAds from chimpanzees and gorillas, belonging to different genetic groups. Among these, Group C adenoviral vectors stand out as the most potent inducers of T cell responses, owed to their lower induction of innate immunity (type 1 IFNs) and higher, prolonged antigen expression. GRAd-COV2, a GRAd-based COVID-19 vaccine, has been the first product based on this platform technology, tested in humans and demonstrated to induce a potent and broad $CD8^+$ and Th1-skewed $CD4^+$ response, associated to a good safety profile and elevated productivity. Building on the success of GRAd-COV2, we are now leveraging the GRAd vector technology to develop a potent T cell inducing HIV vaccine, relying on an innovative T cell immunogen design based on mutationally constrained $CD8^+$ T cell epitopes (GrAdHIVNE1). These epitopes were selected from several clade B and C HIV proteins by structure-based network analysis, are commonly targeted by highly functional $CD8^+$ T cells in HIV elite controllers and provide broad coverage of prominent HLA class I alleles. Preclinical immunogenicity in inbred and mono-allelic HLA-A*0201 knock in mice have confirmed the ability of GrAdHIVNE1 to successfully induce high magnitude, broad and functional $CD8^+$ T cell responses, and the GRAdHIVNE1 vaccine manufacturing and release for the IAVI C-114 phase I clinical trial scheduled for Q1 2025 in Southern Africa are underway. This innovative approach underscores GRAd's potential as a versatile and potent vaccine platform for addressing global health challenges.

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OR037

Liver-directed AAV gene transfer corrects hypoglycemia and metabolic impairment in a GSDIII mouse model in the long term

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Glycogen storage disease type III (GSDIII) is a rare autosomal disorder due to mutations in the gene encoding for glycogen debranching enzyme (GDE). GDE is involved in glycogenolysis, and its deficiency leads to abnormal glycogen accumulation especially in muscles and liver where glycogen metabolism is central for energy supplies. Patients present with fasting hypoglycemia and an evolving liver disease characterized by hepatomegaly, fibrosis, and tumor development. With aging, the progressive muscular weakness worsens, leading to mobility impairment. Most patients also develop cardiomyopathy. To date, no curative treatment is available. Patient's regimen is adapted to reduce glycogen accumulation and avoid hypoglycemia (frequent feeding, cornstarch, and/or nocturnal gastric feeding), and to prevent cardiomyopathy.

The main limit to use AAV-based gene transfer for GSDIII is the size of the GDE coding sequence which exceeds the AAV packaging capacity. We recently reported a dual vector approach to treat the liver in GSDIII mice. However, the use of dual vector for systemic injection is complicated by the increased dose and by the possibility to generate truncated proteins. An additional challenge shown in the previous study was the difficulty to treat the liver in this pathology that required high vector doses and resulted in a variable correction of this tissue. A single vector approach based on the expression of the GDE transgene specifically in the liver demonstrated only transient efficacy in GSDIII mice. To overcome these challenges, we generated an optimized single AAV vector approach based on modifications of the transgene sequence, an improved AAV vector and an engineered, short liver-specific promoter.

Treatment of GSDIII mice with the optimized vector demonstrated correction of hypoglycemia and liver impairment at 9 months after vector injection. Interestingly, CpG dinucleotides content reduction in the transgene expression cassette resulted in longer stability of transgene expression and correction of the metabolic phenotype suggesting a role for innate immunity in the observed transgene expression loss. Evaluation of the mechanisms underlying the reduced persistence observed in this mouse model identified the most prominent as increased liver proliferation with a subsequent AAV genome methylation, an unbalance in the non-parenchymal cells in GSDIII livers and a non-canonical immune response to the AAV vector.

To conclude, our work supports the feasibility of an AAV gene transfer for the correction of liver disease in GSDIII and shows that the observed variability of efficacy and persistence observed in GSDIII livers after AAV gene transfer is due to the underlying metabolic disease. In a broader perspective, this study pinpoint GSDIII mice as a good model to evaluate host-vector interactions in the context of a subclinical liver disease. It also highlights the need to investigate in detail the liver condition in patients enrolled in liver-targeted gene therapy clinical trials, to tailor

immunosuppressive strategies to the underlying liver disease and provide higher chances to patients to achieve stable and efficient gene transfer.

OR038

Safe and effective liver-directed AAV-mediated homology-independent targeted integration in mouse models of inherited diseases

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Liver-directed gene transfer using adeno-associated viral (AAV) vectors holds promise for treating liver and systemic diseases. However, the non-integrative nature of AAV vectors limits their application to newborn livers which is necessary for treating conditions like early-onset inborn errors of metabolism.

To overcome this shortcoming, we developed CRISPR/Cas9 AAV-mediated homology-independent targeted integration (HITI) of therapeutic transgenes at the 3' end of mouse Albumin locus which is highly expressed in hepatocytes.

We show the effectiveness of AAV-HITI in two different mouse models of inherited diseases, Haemophilia A (HemA) and Mucopolysaccharidosis type VI (MPS VI) in which we achieve stable therapeutic levels of systemic proteins, even at low AAV doses. Moreover, we extensively characterised the landscape of HITI donor DNA integrations at the on-target, demonstrating partial AAV genome integrations as well as full length HITI donor DNA which is preferentially cleaved from the flanking ITRs as expected based on its design.

In addition, within the 1-year follow-up no gross chromosomal rearrangements and negligible insertions/deletions events were found at off-target sites. In line with this, no evidence of hepatocellular carcinoma was observed. Finally, we show that AAV-HITI is effective also in adult MPS VI or HemA livers, at vector doses considered safe if translated to humans. Overall, our data support AAV-HITI as a potential therapeutic option for the treatment of those conditions which involve the liver as target tissue.

OR039

Pre-clinical development of an ex-vivo lentiviral-based Hematopoietic Stem/Progenitor Cells-Gene Therapy (HSPC-GT) for Mucopolysaccharidosis type IVA (MPSIVA) as part of an innovative GT platform approach for LSDs with skeletal involvement

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Mucopolysaccharidosis type IVA (MPSIVA) is a lysosomal storage disease (LSD) caused by a deficiency of N-acetylgalactosamine-6-sulfatase (GALNS), characterized by severe skeletal dysplasia, for which current therapies are ineffective. Our study aims to develop hematopoietic stem progenitor cell-gene therapy (HSPC-GT) for MPSIVA as part of an innovative GT platform approach for LSDs. This therapeutic approach is based on preclinical and clinical data demonstrating the beneficial effects of HSPC-GT on the skeletal disease of Mucopolysaccharidosis type I-Hurler (MPSI-H) patients (1-3). To this aim, we have generated a third-generation lentiviral vector encoding for the GALNS enzyme (LV-GALNS), using the same LV backbone employed in MPSI-H clinical trial (ClinicalTrials.gov, NCT03488394) to transduce human HSPCs. The transduced cells showed proper clonogenic and proliferative capabilities in vitro and significantly released the therapeutic enzyme. Using conditioned medium from LV-GALNS transduced myeloid cultures, we restored GALNS expression and activity in patient-derived fibroblasts, mesenchymal stromal cells (MSCs), and MSC-derived osteoblasts. Importantly, osteoclasts derived from LV-GALNS transduced HSPCs secreted GALNS, possibly functioning as a resident source of enzyme for skeletal cross-correction. When transplanted in sub-lethally irradiated NSG mice, LV-GALNS HSPCs efficiently engrafted and repopulated the hematopoietic system. Notably, we detected significantly higher levels of GALNS enzyme in the bone marrow of mice treated with HSPC-GT. Additionally, we tested the clinical-grade LV-GALNS in human HSPCs to determine the most effective transduction protocol for future manufacturing processes. Overall, our preclinical results show efficient transduction of human HSPCs without signs of toxicity, with the hematopoietic progeny of transduced cells releasing supranormal levels of the therapeutic enzyme, capable of metabolically cross-correcting patient cells. To test the superior efficacy of HSPC-GT compared to standard hematopoietic stem cell transplantation (HSCT), we have recently generated a Galns knock-out (-/-) mouse model using CRISPR-Cas9 technology, which shows signs of skeletal disease, including shorter bones, reduced mineral density, and osteoblast number, in addition to a swollen, disorganized, and inflamed growth plate structure compared to age- and sex-matched wild-type controls. On the newly developed MPSIVA mouse model, we have initiated a GT proof-of-concept study including MPSIVA mice transplanted with wild-type or LV-GFP Galns-/- HSPCs and MPSIVA untreated mice as controls. Preliminary results

show proper engraftment of Galns-/- HSPCs transduced with LV-GALNS and improved GALNS activity, reaching supraphysiological levels in PBMNCs from GT treated mice compared to controls. Further evaluations at endpoint will reveal the effects of the therapy the metabolic and skeletal parameters of the disease. These findings will implement our data, supporting the clinical development of HSPC-GT for MPSIVA treatment.

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OR040

Domain-substituted IGF2 tag to modulate receptor targeting during lentiviral gene therapy for Mucopolysaccharidosis type II

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Hematopoietic Stem and Progenitor Cell-mediated Lentiviral Gene Therapy (HSPC-LVGT) provides a promising, potentially lifelong treatment option for (neuro)metabolic diseases such as Pompe disease and Hunter syndrome. At LentiCure – our recently established startup – we are dedicated to bringing these therapies to the market for a reasonable and transparent price. To improve efficacy in muscle and brain, we have developed enhanced versions of HSPC-LVGT utilizing IGF2-tagging. This strategy proved extraordinary effective in preventing muscle and brain pathology in murine models for Pompe and Hunter disease, respectively. Yet, sustained IGF2 expression may disrupt glucose homeostasis due to its binding to the insulin receptor (IR). Here, we present a novel domain-substituted IGF2 insertion-deletion design (IGF2indel) in which domain C and parts of domains A and B of mature human IGF2 are substituted with exogenous epitopes to reduce IR binding and target receptors other than those normally bound by IGF2. When fused to IDS (iduronate-2-sulfatase) – deficient in Hunter disease – IGF2indels incorporating ApoE2 and RAP12x2 inserts demonstrated high-affinity binding to LRP-1 (for brain delivery) and CI-M6P/IGF2R (for lysosomal delivery), while showing no affinity for IR. In *Ids*$^{y/-}$ mice (a model for Hunter disease), HSPC-LVGT with IDS.IGF2indel variants effectively corrected pathology in liver, spleen, heart vessels, valves, and bone microarchitecture, but not cartilage. Importantly, IDS.IGF2indel variants prevented brain heparan sulfate accumulation and inflammation more effectively than untagged IDS and at levels comparable to those of IGF2-tagged IDS. These findings introduce IGF2indel as a novel tag design that enhances safety and efficacy in IGF2-based therapeutics, and present novel candidate transgenes for HSPC-LVGT in Hunter disease.

OR041

Selective transduction of lymphoid cell subsets with bispecific DART-AAVs

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Gene delivery by AAVs as vector platform is facing the problem of unspecific gene transfer due to the broad tropism of the wild type capsid. Therefore, AAV based *in vivo* gene delivery is limited to local administration or the liver as target organ. Accordingly, cells of the lymphoid lineage such as CD8+ T lymphocytes, HIV reservoir cells or NK cells have not been target cells for AAV vectors so far. Here we describe bispecific DARPin-targeted AAVs (DART-AAVs) showing specific transduction of lymphoid cell subsets with bispecific DART-AAVs exhibiting a logic AND-gate-like behavior in receptor usage and target cell selectivity.

For an efficient retargeting of AAV2 vectors, either one or two DARPins were incorporated into the GH2/GH3 loop of VP1, without altering the core structure of the vector capsid. Interestingly, bispecific DART-AAVs targeting CD4 and CD32a consistently showed preferential transduction of double positive cells in whole blood assays as well as *in vivo* after systemic administration. The preference for double positive cells reached up to a factor of 30 compared to single positive cells. Based on these results, the bispecific DART-AAV platform was further expanded towards T cell and NK(T) cell targeting by using previously established CD8-specific or *de novo* selected CD56-specific DARPins. Combining the CD4 with the CD8 DARPin on the AAV2 capsid enabled binding and transduction of the whole T cell population however with a clear preference for CD4/CD8-double positive cells. Bispecific DART-AAVs targeting CD8/CD56 double-positive cells were able to specifically bind their target cells in freshly isolated PBMCs, with the strongest binding signal on CD8/CD56 double positive cells. These CD8/CD56 bispecific DART-AAVs were additionally capable of transducing primary NK cells with strongly enhanced transduction in comparison to their monospecific counterparts.

Our results suggest that simultaneous targeting of two cell surface markers enables selective gene transfer into subpopulations of cell types which so far were not targetable. Additionally, bispecific DART-AAVs increase transduction efficiencies on hard to transduce cell types such as NK cells.

OR042

Development of engineered AAV variants to target T lymphocytes in vivo

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Adoptive T cell therapy using chimeric antigen receptor (CAR)-T cells or recombinant T cell receptor (rTCR) T cells is an accepted treatment strategy in oncology and has expanded to non-

malignant diseases such as fibrosis with promising first reports on therapeutic efficacy. However, the current standard procedure relies on *ex vivo* modification of autologous cells followed by re-infusion, a process that is complex, expensive and time consuming. *In vivo* modification of T cells seeks to overcome these limitations by augmenting T cell viability and function, further expanding applications for adoptive T cell therapy. Adeno-associated virus (AAV) vectors are potent gene delivery tools for *in vivo* gene therapy of numerous inherited and acquired diseases. Capsid-engineering strategies can be applied to vector capsids to improve efficiency of gene delivery, and to re-direct and restrict vector tropism towards defined target cell types. The aim of this project is to develop AAV capsid variants that selectively transduce $CD4^+$ and $CD8^+$ T lymphocytes *in vivo*.

An AAV serotype 2 (AAV2)-based peptide display library was administered intravenously to non-human primates (NHP) and selected for variants with tropism for T cells. Viral DNA isolated from defined rhesus macaque T cell populations was analysed by next-generation sequencing (NGS). The 21 most enriched variants were chosen for further characterization. These variants were produced as vectors encoding the enhanced green fluorescent protein (EGFP). *Ex vivo* transduction experiments on HEK293 cells and primary human $CD4^+$ and $CD8^+$ T cells verified T cell retargeting for 12 of 21 variants. These variants also outperformed parental AAV2 T cell transduction efficiency, with Vr13 transducing 30% and 56% of $CD4^+$ and $CD8^+$ GFP positive cells, respectively, compared to 14.7% and 29.9% in case of AAV2. These 12 variants were then produced with a barcoded EGFP and directly compared for transduction efficiency and cell type selectivity in human whole blood. NGS analysis of the T cell and non-T cell populations revealed that all variants transduced T-cells with greater efficiency than the parental serotype. Although AAV6 vectors showed higher transduction efficiency, this serotype was unable to discriminate between on-target and off-target cells. Our variants Vr02 and Vr03 demonstrated respective target:noise ratios of 4.7 and 4.3 compared to 0.9 for AAV6. Based on these promising data, we performed a biodistribution analysis in wild type C57BL/6N mice. Vector genome and transgene expression assays are currently underway to quantify modification of our target cell population as well as for common off-target tissues. These experiments will evaluate the T cell targeting capacity of our refined pool of T cell tropic variants following intravenous administration.

OR043

Investigating the safety, efficacy and dynamics of lentiviral and AAV gene therapies in a mouse model of maple syrup urine disease

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Maple syrup urine disease (MSUD) is an inborn error of metabolism characterised by deficient metabolism of branched chain amino acids (BCAAs), owing to mutations afflicting any of the constituents of the branched chain ketoacid dehydrogenase complex. It is associated with acute encephalopathy and early death if untreated, and its treatment options are limited to BCAA dietary restriction, haemofiltration and liver transplantation. We are investigating gene therapies for this condition using VSVg-pseudotyped lentiviral (LV) and adeno-associated viral 9 (AAV9) vectors. Both vectors encode a codon-optimised human DBT transgene under the control of a synthetic promoter, and have been administered to pups at P1-P2 intravenously.

To arrive at safe dosages that can be progressed for efficacy assessment, a 120-day dose-finding toxicity study was conducted in wildtype mice. LV was tested at doses of 4.8e10 TU/kg (n=9) and 1.4E11 TU/kg (n=7), and AAV9 at 8.7E13 vg/kg (n=8) and 4.4E14 vg/kg (n=5). All LV and AAV9 treated mice lived to P120, except for one high-dose LV animal that survived to P83. Systemic toxicity analyses in the forms of weight gain, behavioral and liver H&E assessments rendered unremarkable results, as did the plasma levels of alanine aminotransferase, aspartate aminotransferase, bilirubin, albumin, creatine kinase and creatinine. Hepatic transduction was confirmed with both vectors via qPCR, wherein vector copy number (VCN) and transgene expression levels correlated with dose.

Efficacy of these vectors was tested in a mouse model of MSUD, wherein the endogenous *Dbt* (*mDBT*) gene is knocked out. The LV and AAV doses employed respectively were 1.4E11 TU/kg (n=9) and 1.0E14 vg/kg (n=9). Mice were sustained on diets with normal content, and the ataxic phenotype was assessed at p120 via catwalk analysis. All wildtype controls (n=9) survived to P120, whereas untreated knockouts (n=20) respectively had median and maximal survivals at P20 and P36. AAV9-treated subjects had a median survival at P73, and only one survived to P120. In contrast, seven LV-treated animals survived to P120. These LV-treated animals demonstrated correction of speed (15.2±1.2 vs 15.0±0.6 cm/s; p>0.9999), hind limb swing speed (52.0±1.9 vs 53.6±1.2 cm/s; p=0.9712), stride length (5.0±0.2 vs 5.5±0.2 cm; p=0.1839) and regularity index (89.3%±0.7% vs 90.0%±1.6; p>0.9999) compared to wildtypes respectively.

Molecular analyses focused on AAV9 (n=6) and LV (n=9) treated knockouts that survived past P36, thereby outliving the longest-lasting untreated knockout. LV and AAV9 treated subjects had plasma BCAA:alanine ratios that were around fivefold higher than those of post-weaning wildtypes (n=8) (4.3±0.2 vs 4.1±0.3 vs 0.9±0.1 respectively, p<0.0001). Untreated knockouts (n=4) in contrast exhibited a 25-fold elevation over age-matched wildtypes (n=5) (7.6±0.7 vs 0.3±0.1, p<0.0001). Despite LV having a significantly lower hepatic VCN compared to AAV9 (0.7±0.1 vs 9.2±2.6 vector copies per diploid genome, p=0.0219), it mediated a transgene expression level that was fivefold higher (32%±5% vs 6±1% of mean wildtype *mDBT* mRNA level, p=0.0029). This study validates the utility of systemic LV gene therapy for the treatment of MSUD, and paves the way for investigations into the effects of transgene depletion and epigenetic silencing on hepatic AAV9 and LV transgene expression.

OR044

AAV(BBB)s: Novel AAV Variants with 500-fold Higher BBB Crossing Efficiency in NHP

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Gene therapy targeting CNS diseases faces significant challenges primarily due to the blood-brain barrier (BBB), which restricts the entry of therapeutic delivery vehicles. Adeno-associated virus serotype 9 (AAV9) has become a focal point in CNS gene therapy due to its ability to cross the BBB in mouse models. AAV9 serves as a foundational template for developing new AAV vectors with enhanced BBB penetration capabilities, such as AAV-Php.eB. However, AAV capsids that were modified for BBB crossing in mice have shown limited success in non-human primates (NHPs). To explore potential mechanisms, we systematically assessed commonly used AAV serotypes (AAV1-AAV13), known BBB-crossing variants, and other popular engineered capsids

using our ATHENA I platform, which incorporates DNA barcode technology. Our findings confirmed that AAV9 remains the superior template for BBB-crossing selection in B6C3 mice. Variants like AAV-9P31, AAV-F, AAV-Php.C2, and AAV-Php.eB were the most effective in most brain tissues. Nonetheless, in NHP models, AAV9 variants did not exhibit enhanced BBB crossing capabilities and showed very low DNA levels compared to mouse models. Using our ATHENA I platform, we identified a novel AAV capsid, termed AAV(BBB), which displayed a remarkable approximately 140-fold increase in DNA accumulation in the NHP brain relative to AAV9, and double the RNA levels. To further enhance RNA expression, we evolved this capsid using our ATHENA III DNA shuffling platform. The resulting AAV(BBB) variants demonstrated up to a 500-fold increase in DNA and/or a 10-fold increase in RNA levels across the BBB in NHPs, compared with AAV9. Moreover, some AAV(BBB) variants showed a specific affinity for the NHP brain, with significantly reduced DNA accumulation and RNA expression in other organs. This breakthrough underscores the potential of AAV(BBB) variants as promising candidates for CNS gene therapy, setting the stage for further research and development in this promising area.

OR045

Towards the translation of an AAV-mediated gene therapy for an incurable disease

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CTNNB1 Syndrome is a rare neurodevelopmental disease affecting (1:50,000 births) caused by mutations in *CTNNB1* gene encoding beta-catenin. Mutations in this gene can lead to the production of a decreased amount of the functional protein or a defective protein with decreased activity. Beta-catenin is essential for neuronal development, synapse formation, and maturation of the brain. Consequently, mutations in the *CTNNB1* gene can lead to cognitive impairments such as intellectual disability, learning difficulties, and developmental delay. Additionally, CTNNB1 Syndrome is characterised by progressive spasticity and motor skills deterioration.

While there is currently no treatment for CTNNB1 Syndrome, the genetic root cause of the disease could be addressed with recombinant adeno-associated viral (rAAV) vector-based gene augmentation strategy. This strategy aims to deliver an additional functional copy of the gene to the affected cells involved in disease phenotype, with the aim to recover the normal protein functions and the potential amelioration or even correction of the disease.

As no gene augmentation therapy had been tested before for this disorder, our group designed six different AAV-CTNNB1 constructs (C1-C6). Each construct included the coding sequence of *CTNNB1* gene along with a variety of untranslated regulatory elements selected to enhance the

expression of the gene in the target cells and inhibit expression in off-target tissues. The regulatory elements included fragments of the endogenous gene such as UTRs or intronic sequences, as well as other post-transcriptional regulatory elements such as microRNA targeting sequences (miRTS).

The therapeutic constructs were individually packaged into AAV vectors, which were subsequently used to transduce patient-derived neuro-progenitor cells and cortical brain organoids. We found that only one of the constructs, AAV-CTNNB1(C4), was able to restore beta-catenin expression levels and function in both preclinical models. The AAV-CTNNB1(C4) construct underwent further functional analysis in a disease mouse model (*Ctnnb1*$^{+/-}$). To do so, three doses (low= 4.24e9, mid= 2.12e10, high= 1.06e11 vg/mouse) of an AAV9-CTNNB1(C4) vector were inoculated intracerebroventricularly (ICV) into 4-week-old animals, and behavioural tests were conducted to assess phenotype correction. At 16 weeks post treatment, we observed correction of anxiety and locomotor functions, two phenotypes that recapitulate symptoms manifested by CTNNB1 Syndrome patients. Animals were sacrificed at 20 weeks of age to confirm the expression of human beta-catenin in different regions of the brain at mRNA and protein levels. In addition, the lack of target expression in off-target tissues, such as the liver, served to verify the efficiency of the miRTS.

Critically from the translational perspective, we also confirmed safety of the AAV-CTNNB1(C4) vector in 4-week-old C57Bl/6 mice that were ICV inoculated with 1.06e11 and 2.25e11 vg/mouse and monitored for 8 months.

In conclusion, our group developed the first AAV-based gene therapy for CTNNB1 Syndrome which resulted in the correction of beta-catenin expression and function in preclinical models. Our strategy was able to correct some of the disease phenotypes reported in patients while being safe at the tested doses. These results support an investigational gene therapy clinical trial to treat CTNNB1 Syndrome that is expected to start in Europe and Australia in Q2 2025.

OR046

Critical role of VP3 N-terminal residues and variable capsid regions for bocaparvovirus transduction

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The last decade has seen a tremendous progress in adeno-associated virus (AAV)-mediated gene therapy evidenced by the steep rise in the numbers of drug approvals and clinical trials. Nevertheless, the application of AAV gene therapy is limited due to AAV's small packaging capacity (~4.7 kb) as well as its tendency to transduce off-target organs and tissues. A previous study from our group has demonstrated that several bocaparvovirus (BoV) variants with a packaging capacity of ~6 kb can transduce various human primary cells. Other groups have also reported recombinant human bocavirus 1 (HBoV1) transduction in lung *in vivo*, supporting the great potential of BoV as an alternative gene therapy vector. However, a high dose is usually required for effective gene delivery *in vivo*, and the transduction efficiency *in vitro* (i.e. cell lines) tends to be lower than that of most AAV isolates. With the aim of improving BoV transduction efficiency, we explored amino acid residues on the capsid protein that may be important for transduction. Specifically, segments of gorilla bocavirus 1 (GBoV1) were replaced to corresponding residues from HBoV1, as GBoV1 exhibits significantly higher transduction efficiencies on multiple cell lines despite the high amino acid identity of these two bocavirus

variants (86%). Intriguingly, luciferase analysis *in vitro* showed that a short stretch of amino acids at the N terminus of VP3, but neither VP1u nor VP1/2 common N-terminal regions, are at least partially responsible for the difference in transduction efficiency of the two variants. Applying this new finding, we mutated the residues of the HBoV1 VP3 N terminus to the corresponding residues in GBoV1, which successfully enhanced HBoV1 transduction efficiency about two-fold over the wild-type without reducing viral vector production. Additionally, our comparison of the structures of bocavirus variants to AAV revealed a unique structural feature in bocavirus, namely, a relatively short variable region VIII (VRVIII) and its close distance to VRIV. Further sequence and structure alignments among bocavirus capsid variants implied an interaction of conserved residues on the two VRs. Notably, while individual mutations on VRIV or VRVIII moderately enhanced GBoV1 transduction efficiency, concurrent alteration of residues in both loops enhanced transduction about 2.5-fold over the wild-type. A similar pattern was observed for HBoV1, albeit to a lesser extent. To the best of our knowledge, this is the first study suggesting the importance of the structurally flexible and unresolved region of the bocavirus capsid, as well as of its surface regions for cell transduction. Studies are underway to investigate the underlying molecular mechanisms and to explore the efficacy of the engineered capsids *in vivo*.

OR047

Efficient base and prime editing to correct COL7A1 patogenic variants in primary RDEB patient keratinocytes

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Recessive dystrophic epidermolysis bullosa (RDEB) is a severe genetic disorder caused by bi-allelic pathogenic variants in the *COL7A1* gene, which encodes type VII collagen (C7), including nonsense and frameshift mutations. Among non-DNA disruptive therapeutic genome editing (GE) strategies, adenine base editors (ABEs) and prime editing are particularly suited and effective to correct these kinds of mutations. Here, we evaluated the efficacy and precision of these technologies to correct three different *COL7A1* mutations in primary patient keratinocytes. We achieved over 90% efficiency using ABE8e, delivered via mRNA electroporation, for mutations located in two different exons (72 and 94). Editing on the intended nucleotide and the adjacent thymine (generating a silent mutation) was achieved. The restoration of C7 expression in treated cells corresponded with genotypic correction. Skin equivalents composed of base-edited keratinocytes and fibroblasts from the patient were generated. This bioengineered skin demonstrated long-term engraftment in immunodeficient mice. Moreover, in vivo regenerated skin regeneration showed adequate C7 deposition at the basement membrane. In order to explore an alternative approach capable of performing a wider range of DNA modifications, we assessed prime editing to correct one of the most prevalent *COL7A1* frameshift mutations (c. 6527insC) restoring the wild-type sequence. Using optimized prime editors (PEmax) and engineered prime-editing guide RNA (epegRNA), we report over 50% correction efficacy in primary keratinocytes from four different RDEB patients. Immunofluorescence analysis showed C7 expression in treated cells comparable to healthy ones. In vivo experiments are underway. In conclusion, we show for the first time that ABEs and prime editing targeted to keratinocytes can be highly effective and safe gene correcting modalities, expanding the genome editing treatment options to cope with the large *COL7A1* mutational spectrum causing RDEB.

OR048

Biofabrication of a hyaline cartilage graft mimicking native architecture from genetically engineered MSCs with enhanced chondrogenic potential

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Hyaline cartilage is a connective tissue characterized by a distinctive aligned zonal architecture that confers significant resistance to the mechanical loading experienced by articular joints. However, its avascular, aneural and alymphatic nature hampers its regeneration ability upon injury, often leading to osteoarthritis. While tissue engineering approaches are emerging as promising methods to restore the function of injured tissues, generating engineered cartilage constructs with native-like properties remains a significant challenge. Specifically, the development of cellular cartilage grafts is hindered firstly by the tendency of chondrocytes to dedifferentiate during the *in vitro* expansion and secondly by the difficulty in precisely recapitulating the zonal architecture of cartilage, including the orientation of collagen fibrils. Mesenchymal stem cells (MSCs) hold promises for cartilage regeneration due to their enhanced proliferation and differentiation potential, their potential use for autologous therapeutics and their immunomodulatory properties. However, the neocartilage formed by MSCs is unstable, as it undergoes hypertrophy and extracellular matrix calcification, ultimately leading to endochondral ossification, a developmental pathway that results in bone formation. In this study, we used a clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) ribonucleoprotein (RNP) complex to knockout (KO) the key osteogenic transcription factor Runt-Related Transcription Factor 2 (RUNX2) in a primary human MSCs cell pool. Furthermore, we combined edited MSCs with Filamented Light (FLight) Biofabrication, a bioprinting technique capable of producing highly aligned tissue constructs that mimic the architecture of articular cartilage. We achieved a striking *95% RUNX2* bulk KO efficiency in primary cells, while simultaneously preserving the MSCs' proliferation and differentiation potential. Remarkably, histological analysis and micro-computed tomography revealed that cartilage from KO MSCs was resistant to hypertrophy *in vitro*, a phenomenon which was linked to significantly reduced transcription of genes encoding proteins involved in matrix mineralization and osteogenesis, such as Sp7 Transcription Factor (*SP7*), Secreted Phosphoprotein 1 (*SPP1*) and Bone Gamma-Carboxyglutamate Protein (*BGLAP*). Additionally, KO cells exhibited robustly enhanced chondrogenesis compared to their wild-type (WT) counterparts, secreting significantly greater levels of glycosaminoglycans, and showing increased expression of the chondrogenic master transcription factor SRY-Box Transcription Factor 9 (SOX9). Our engineered grafts matured with highly aligned collagen fibres deposition and displayed mechanical properties approaching the values of the native tissue. Our pioneering work demonstrates the modulation of primary human MSCs cell fate towards stable chondrogenesis using RNP-based gene editing, a strategy that ensures reduced immunogenicity and a low risk for off-target effects. Furthermore, the combination of edited cells with FLight bioprinting technique enabled us to effectively engineer a stable hyaline cartilage graft that preserves the native tissue microarchitecture, making it suitable for clinical applications.

OR049

iqDNA is an engineered DNA cargo that avoids innate immune activation while retaining durable transgene expression

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Non-viral delivery of large DNA molecules offers the potential for long-lasting, redosable, gain of function genetic medicines, and may broaden the scope of *in vivo* gene editing to support full gene correction. One of the central challenges to realizing this enormous potential is the acute DNA-mediated activation of the innate immune system.

Immune quiet DNA (iqDNA) is a new class of gene therapy cargo engineered to avoid activation of Pattern Recognition System (PPR) systems (e.g., cGAS/ STING, TLR9, Inflammasome), while retaining the capacity to support episomal transgene expression. A key structural feature of iqDNA is the minimization of double-stranded (ds) DNA regions known to preferentially stimulate cGAS, in addition to close-ended DNA termini that prime host-dependent conversion of iqDNA into a transcriptionally active dsDNA molecule in the nucleus.

When iqDNA is administered systemically using lipid nanoparticle delivery, mice show baseline levels for a broad range of cytokines across a dose range. This profile is comparable to chemically modified mRNA and stands in contrast to the high cytokine stimulation observed for dsDNA formats. Critically, the immune quiet profile of iqDNA is robust across species, avoiding innate immune activation in mice, non-human primates, and human PBMCs. Importantly, iqDNA also supports robust and durable expression. Mice treated with a low dose of LNP encapsulated iqDNA encoding a luciferase reporter showed similar expression to a dsDNA analogue (ceDNA) and was maintained though 30 days (~ 10^8 ph/s, @ 0.25 mg/kg, *i.v.* delivery)

Efforts to optimize potency of iqDNA have centered on improving resistance to enzymatic degradation and promoting conversion to a transcriptionally active molecule. To this end, our iqDNA manufacturing process, Rapid Enzymatic Synthesis (RES), enables flexible modification of any DNA sequence element, including incorporation of modified bases, structured elements, and handles for bioconjugation. We show that inclusion of DNA modifications at the 3' and 5' free ends of iqDNA improves resistance to degradation by the exonuclease Snake Venom Phosphodiesterase (SVPD). Additionally, we articulate design principles of iqDNA governing gene expression, demonstrating how encoding regions of the promoter as a dsDNA element significantly enhances transgene expression *in vivo*, while still avoiding activation of the innate immune system.

In support of its therapeutic potential, iqDNA encoding a ~ 7kb FVIII transgene cassette avoided stimulation of the innate immune system in mice and demonstrated persistent hFVIII expression. We are in the process of incorporating performance optimizations into FVIII iqDNA and translating this work into NHP.

The tolerability profile of iqDNA paired with its ability to support therapeutic transgene expression represents an important step toward enabling functional, durable, and redosable non-viral genetic medicines.

OR050

tLNPs can effectively deliver DNA to T-cells and generate long-acting CAR-T cells *in vivo*

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Ex-vivo modification of immune cells to express Chimeric Antigen Receptor (CAR) has shown tremendous clinical and commercial success as a cancer treatment. Although widely adopted, *ex-vivo* CAR-T approaches are not without their challenges. Soaring production expenses, extended timelines, inherent toxicity risks and the operational intricacies of today's conventional cell therapy treatment models calls for a next wave of improvements.

Here we present a novel cell-targeted lipid nanoparticle (tLNP) that can deliver both DNA and RNA to T cells. This new tLNP based vector consists of an LNP formulation containing transposon DNA (encoding for CAR), mRNA (encoding for transposase) and activating and targeting protein moieties. We will discuss the ability of this vector to activate resting primary T cells, thereby allowing their permanent modification with a DNA encoded CAR construct *in vitro* in the absence of exogenous activation resulting in the generation of fully functional CAR-T cells. In addition, the capacity of this non-viral vehicle to drive the generation of functional T-cells *in vivo* will be presented. We will demonstrate the generation of persistent CAR-T cells, tumour control and extended survival after tLNP treatment in a human PBMC engrafted NSG mouse model injected with a human B-cell leukaemia cell line.

We believe that the CAR-T cells generated *in vivo* have a variety of advantages compared to currently employed ex-vivo manufacturing technologies.

OR051

Exon skipping using palmitoyl conjugated tricyclo-DNA antisense oligonucleotides restores protein expression *in vivo* in recessive dystrophic epidermolysis bullosa

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Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a rare hereditary genodermatose characterized by detachment of the skin and mucous membranes, with serious local and systemic complications, for which no curative treatment is available. It is caused by loss-of-function variants in *COL7A1* encoding type VII collagen (C7), the major component of anchoring fibrils which are essential structures for dermal-epidermal adhesion. More than 1200 pathogenic

variants have been identified in *COL7A1*, which contains 118 small exons (NM_000094.3). Of these, 82 consecutive exons are in frame and encode the central helical domain that can potentially be shortened without affecting the protein function, making it an promising target for antisense oligonucleotide (ASO)-mediated exon skipping. We have developed a palmitate-conjugated tricyclic-DNA ASO (Palm-tcDNA) targeting the frequently mutated exon 73. Transfection of this ASO in primary keratinocytes and fibroblasts from RDEB patients induces efficient exon 73 skipping and re-expression of C7 as demonstrated by Western Blot and immunofluorescence analyses. To demonstrate the feasibility of the approach on human *COL7A1* transcripts *in vivo*, this ASO was subcutaneously and intravenously injected in a transgenic murine model (m*Col7a1*$^{-/-}$;Tgh*COL7A1*) carrying the entire human *COL7A1* genomic locus. Analysis of transcripts from tissues usually affected in RDEB (skin, eye, and oesophagus) revealed variable levels of exon 73 skipping *in vivo*, consistent with the results of ASO biodistribution. Reconstructed skin from C7-deficient RDEB patient cells was generated and grafted onto an immunodeficient murine model (NMRI-*Foxn1*$^{nu/nu}$). We demonstrated restoration of C7 re-expression at the dermal-epidermal junction up to one month after the last injection following either two subcutaneous injections of 500 µg or 1 mg of the ASO under the graft, or 4 or 8 weekly intravenous injections at 50mg/kg. No adverse reactions were observed in treated mice. Formation of anchoring fibrils was also assessed by transmission electron microscopy. *In vitro* pre-toxicology studies showed that the ASO do not form homodimers nor induce plasma aggregation nor activate complement, which would have precluded systemic administration in human. Overall, our data indicate that systemic administration of palmitoylated tricyclo-DNA ASO restores C7 expression by skipping exon 73 of *COL7A1* pre-mRNA and holds therapeutic potential for RDEB patients.

OR052

Optimization of in vitro and in vivo performance of circVec, a vector-based circular RNA expression platform for enhanced gene therapy

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Circular RNA (circRNA) is a class of endogenously expressed non-coding transcripts found in most eukaryotes. In contrast to mRNA, circRNA is resistant to the intra-cellular exo-nucleolytic RNA turnover, resulting in increased stability and persistence within cells. With enhanced understanding of circRNA biogenesis and functionality and recent engineering of circRNAs for highly efficient translation of proteins, circRNA is rapidly emerging as a next generation therapeutic RNA format that is expected to outperform conventional linear mRNA.

Circio is developing a novel spliceosome-dependent expression system, circVec, designed and optimized for potent and durable intracellular expression of circRNA from viral and non-viral DNA vectors. Here, we show that protein expressed from the optimized circVec genetic cassette accumulates over time and reaches almost 10x higher levels compared to mRNA-based vectors one week after transient transfection *in vitro*. Using intramuscular injection of DNA vectors in both immune-compromised and -competent mouse models, circVec significantly demonstrates 3-13x increased gene expression compared to the mRNA benchmark.

Circio is deploying the circVec platform to enhance gene therapy for genetic disorders requiring high and durable gene expression, with a lead program in alpha-1-antitrypsin deficiency (AATD). AATD is caused by pathogenic mutations in the *SERPINA1* gene leading to pathology in both lung and liver due to reduced functional AAT levels in circulation and an accumulation of non-functional, toxic AAT protein aggregates, respectively. Circio has developed a unique and bimodal 'remove-and-replace' vector co-encoding a proprietary functional element to specifically target and deplete pathogenic AAT mutant alleles. Simultaneously, the vector delivers high-yield, functional AAT from the circVec cassette. Based on this 'remove-and-replace' cassette design, we show substantially enhanced circular RNA driven AAT levels compared to mRNA-based expression, in parallel with >90% high-specificity knockdown of the mutant AAT form.

These results demonstrate great potential for circRNA-based vector-therapeutics to enhance current gene therapies by achieving higher and more persistent protein expression per vector copy. Therefore, circRNA-based expression systems, such as the circVec platform, can pave the way for new treatment strategies for genetic disorders where conventional gene therapy has so far been unsuccessful.

OR053

Second occurrence of LMO2-associated clonal T cell proliferation 20 years after gamma-retrovirus-mediated gene therapy for SCIDX1

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More than twenty years (Y) ago, we showed correction of X-linked severe combined immunodeficiency SCID-X1, by first generation gamma-retroviral vector (RV) into autologous CD34 bone marrow cells with no conditioning regimen. Nine out of ten patients showed appropriate T cell reconstitution, allowing correction of their disease phenotype. We previously reported that 5 patients experienced serious adverse events (SAE), developing T cell acute lymphoblastic leukemia (T-ALL) after gene therapy (GT) (4 after 2.5–5.6Y and 1 after 15Y), due to integration of the LTR-driven RV near proto-oncogenes. Chemotherapy failed for one patient, but was successful for all other patients. We report here on a patient with a second occurrence of T-ALL, 18 years after the first one. This SAE was diagnosed 20.7Y after GT. Chemotherapy was initiated for the treatment of this thymic T-ALL, but after 9 months due to poor response, HSCT was decided and successfully performed. The patient is doing well 6 months after HSCT.

We showed that this new lymphoproliferation is not a relapse of the previous one and developed from a distinct T-cell progenitor. The therapeutic vector was found in the blast cells, and integration site (IS) analysis disclosed expansion of a clone with IS inside *LMO2* gene (intron2-3, 584bp apart from the insertion responsible for the previous SAE). This IS was detected in the

circulation (PBMC) for the first time at 10.4Y of follow up and remains stable at low frequency (below 1%) till the SAE.

In this patient, we also analyzed IS from cell free DNA (cfDNA) extracted from plasma (using the LiBIS-seq technique). At several timepoints before the occurrence of the T-ALL and till one year before, IS profiles were similar between PBMC and cfDNA with 47% of shared IS and similar major clone in *CCND3* (mean frequency of 37%). Two months before the T-ALL, the frequency of the *CCND3* clone decreased by two-fold in cfDNA and a new IS clone in *LMO2* appeared for the first time at a frequency of 8% in cfDNA whereas in PBMC its frequency was only 0.4%. This clone corresponds to the leukemic clone that expanded in the following 2 months to reach 93% frequency in the circulation at the time of leukemia.

The 5 other patients regularly followed in Necker Hospital (2 SAE, 3 noSAE), are closely monitored. The patient who experienced the SAE 15Y post-GT totally recovered and still presented with a high polyclonal reconstitution, and thymic activity with naïve T cell production, demonstrating the persistence of T-cell progenitor with self-renewal ability. One patient presented with stable oligoclonal reconstitution over 20Y without any SAE. The 3 other patients presented with polyclonal reconstitution (mean Shannon index of 4).

All in all, this second late onset of SAE showed that the genetic dysregulation in the thymus can occur suddenly after a long delay from the first hit, and that IS monitoring in plasma appears to be a powerful and highly sensitive tool for early detection of clonal selection several months before the onset of T-ALL.

OR054

Modeling of VEXAS syndrome by Base Editing uncovers poisoning of healthy hematopoiesis as an unanticipated mechanism driving clonal dominance during aging

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Clonal dominance is common in hematopoiesis during aging and increases susceptibility to malignant and non-malignant diseases. VEXAS syndrome is an acquired, severe autoinflammatory and hematological disease of aging due to clonal dominance of pro-inflammatory cells originating from hematopoietic stem/progenitor cells (HSPCs) bearing a somatic mutation in the *UBA1* gene.

Pathophysiology and mechanisms of clonal dominance in VEXAS remain unclear due to its recent discovery, small and heterogeneous patient cohorts, and lack of experimental models. To address this issue, we leveraged adenine base editing to develop *in vitro* and *in vivo* models of VEXAS syndrome and validated them by multiparametric flow-cytometry and omic analyses on a cohort of VEXAS patients. Base editing in human wildtype HSPCs installed with >90% efficiency the most common *UBA1* mutation (*UBA1*^{mut}). Clonogenic assays of *UBA1*^{mut} HSPCs revealed an exclusive myeloid output *in vitro* compared to wild-type control (*UBA1*^{wt}). Xenotransplantation of *UBA1*^{mut} HSPCs in immunodeficient mice resulted in a 10-50-fold lower human engraftment compared to controls due to dramatic shrinkage of the lymphoid compartment, while the NK and myeloid ones were more preserved. HSPCs were 5-fold lower in number in *UBA1*^{mut} than *UBA1*^{wt} transplanted mice and mostly myeloid-biased, suggesting loss of multilineage repopulating potential and weakened self-renewing capacity. Concordantly, myeloid cells and HSPCs were mostly *UBA1*^{mut}, while differentiated lymphoid cells were only *UBA1*^{wt}. Single-cell RNA-sequencing (scRNA-seq) on the human graft from *UBA1*^{mut} mice revealed pervasive upregulation of inflammatory and apoptotic responses, and lower propensity to engage cell cycle across all hematopoietic subpopulations, particularly of myeloid origin. Primitive HSCs from sorted human HSPCs of *UBA1*^{mut} mice early activate inflammatory responses, are prematurely aged, and transcriptionally imprinted toward myelopoiesis. Notably, the immunophenotype, lineage repopulation patterns, and transcriptomic programs in mice strikingly mirrored those observed in nine VEXAS patients from our cohort, validating the reliability of the model. We then leveraged our humanized model to study how different degrees of mosaicism affect hematopoiesis, and the mechanisms underlying clonal dominance in VEXAS syndrome. Competitive transplants mixing human *UBA1*^{mut} and *UBA1*^{wt} HSPCs at different ratios showed that the absolute number of human cells progressively decreased at increasing input of *UBA1*^{mut} HSPCs, while the myeloid-lymphoid ratio increased. The proportion, but not the absolute number, of *UBA1*^{mut} cells was enriched from the input within HSPCs and myeloid cells at >25% *UBA1*^{mut} HSPCs infused. Conversely, B cells were only *UBA1*^{wt} but dramatically reduced in numbers compared to the input. These data suggested a threshold effect at 25% *UBA1*^{mut} HSPCs above which the pathogenic clone dominates and subverts human hematopoiesis. Intriguingly, scRNA-seq on the bystander lineage-negative murine cells in *UBA1*^{mut} mice revealed potent activation of NFkB-mediated inflammatory signatures, promoting cell proliferation and apoptosis. This data corroborates a mechanism by which the VEXAS clone becomes prevalent and disrupts hematopoiesis by “poisoning” wild-type HSPCs, favoring its dominance over non-mutated cells. In summary, our model faithfully recapitulates the pathophysiology of VEXAS syndrome, providing a novel mechanism driving clonal dominance during aging. Moreover, and is the ideal setting for high-throughput preclinical screening of therapeutic strategies in this novel, common disease.

OR055

In vivo gene transfer into hematopoietic stem and progenitor cells for the treatment of Fanconi Anemia

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Lentiviral vector (LV) mediated *ex vivo* gene therapy in hematopoietic stem and progenitor cells (HSPC) fulfilled the promise of a cure for a number of genetic diseases. However, collection and *ex vivo* manipulation of HSPC and the risks associated with patient conditioning and

transplant still pose challenges to broad access to HSPC gene therapy. These hurdles might be overcome by an *in vivo* approach. To investigate the feasibility of this approach, we studied HSPC biodistribution in newborn, 2-week-old or adult mice and discovered a unique window of opportunity in the formers due to early post-natal persistence of the hepatic foetal hematopoietic niche and extensive trafficking of HSC to the bone marrow (BM). Indeed, we were able to successfully target *bona fide* HSC by intravenous (i.v.) administration of GFP-LV to newborn mice. We obtained stable, life-long GFP expression in up to 10% of all blood lineages, paralleled by a comparable expression in HSPC harvested from the BM, which could engraft long-term in busulfan-conditioned mice. LV integration site analysis confirmed common origin of different hematopoietic lineages from multiple clones. The decrease in HSC number in the circulation observed in 2-week-old mice was paralleled by a decrease in gene transfer efficiency. We thus applied a GCS-F/Plerixafor mobilization regimen, modelling the clinical use, to 2-week-old mice. In these mice, we observed a sharp increase of circulating HPSC and achieved up to 13% GFP-positive cells in the circulation, 10-fold higher than in non-mobilized controls. These data show that the possibility of effectively targeting HSPC *in vivo* can be extended to juvenile mice by applying a mobilization regimen. As a paradigmatic disease model to test this approach we chose Fanconi anaemia (FA), a rare genetic disorder belonging to the DNA repair deficiency syndromes. We administered i.v. FANCA-LV to FA newborn mice and showed a progressive increase of LV-positive cells in treated mice throughout growth and homeostasis of the hematopoietic system, suggesting selective advantage of the corrected cells over the non-corrected ones, coupled with progressive normalization of white blood cell and lymphocyte counts. To mimic the BM failure observed in FA patients, LV-treated FANCA mice were challenged with Mitomycin C (MMC), a DNA cross-linking agent causing death of non-corrected cells. Remarkably, we observed a significant expansion of corrected cells after two doses of MMC, reaching up to 100% in MMC-challenged mice with prompt recovery of blood parameters only in LV-treated mice. Our work shows efficacy and safety of *in vivo* gene transfer into HSPC in mice, life-long maintenance of transgene expression, and, as we also document high numbers of circulating HSPCs early post-natal in humans, set the basis for possible clinical translation of this approach for FA.

OR056

Gene therapy for Recombinase deficient-SCID and hypomorphic RAG disease

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Recombinase-activating gene (RAG) deficient SCID patients lack B and T lymphocytes due to the inability to rearrange immunoglobulin and T-cell receptor genes. The two RAG genes are acting as a required dimer to initiate gene recombination. Gene therapy is a valid treatment alternative for RAG-SCID patients, who lack a suitable bone marrow donor, but developing such therapy for RAG1/2 has proven challenging, given the high expression levels needed, especially for RAG1. To minimize the risks of insertional mutagenesis, we have chosen to aim for VCN around one, to avoid multiple integrations in the same stem cell clone. This preclinical program resulted, surprisingly, in different promoter choice in the LV vectors for RAG1 and RAG2. The vector of choice for RAG1 is currently also tested for diseases with residual RAG activity such as Omenn Syndrome and combined immunodeficiency associated with granulomas and/or autoimmunity (CID-G/AI) with the goal of reaching clinical trial.

Three patients have thus far been included in the RAG1-SCID trial, with encouraging clinical and immunological results. The first patient has fully reconstituted B and T cell repertoire, vaccination responses and development of regulatory T cells, while the second patient showed an interesting gamma delta T cell response, most likely directed against a CMV infection, which was successfully cleared. Integration site analysis indicates a highly polyclonal repertoire, although clones with insertions near known oncogenes also were detected, but at stably low frequencies.

Of note, we aim for multicentre, international trials with various clinical sites in Europe, Asia and Australia. Centres in the UK, Spain, Italy, Poland, Turkey and Australia are nor part of clinical trail network that are allowed to include patients. The stem cells will be modified in one centre (Leiden, Netherlands) and then transported to other clinical sites for transplantation and standardized follow-up.

OR057

Cell-Type and Time-Resolved Genotoxicity of Base Editing

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Base editing is a well-established concept in genetic engineering that is currently being evaluated in clinical trials. It allows the introduction of targeted changes in the genome without the need for DNA double-strand breaks (DSBs). While off-target effects of adenosine (ABE) or cytosine base editors (CBE) have been reported, little is known about their cell type specificity or temporal resolution. To address this issue, we established a CBE-based therapeutic approach in a preclinical model of a hyperinflammatory syndrome, familial hemophagocytic lymphohistiocytosis type 3 (FHL3). FHL3 is caused by mutations in the UNC13D locus and current therapy relies on hematopoietic stem cell (HSC) transplantation. Here, we developed a CBE strategy to disrupt the disease-causing cryptic splice site in Unc13d intron 26 of Jinx mice, a preclinical FHL3 mouse model. Electroporation of CBE-encoding mRNA and gRNA into Jinx T cells and HSCs resulted in 61-71% of edited Unc13d alleles. Genetic and functional assays confirmed correct splicing of the Unc13d pre-mRNA and restored cytolytic activity of the edited T cells. Furthermore, transplantation of Unc13d-edited HSCs into Jinx mice demonstrated functional restoration of lymphocyte cytotoxicity and protection against hyperinflammation in vivo. To evaluate genotoxicity in CBE- and Cas9-edited cells, we used high-throughput sequencing (HTS)-based CAST-Seq in different primary cell types (HSCs, T cells, fibroblasts) to identify chromosomal rearrangements and multiplexed amplicon HTS (rhAmpSeq) to validate off-target (OT) activity. Chromosomal aberrations and OT activity were detected at 28 sites in the three cell types, but not uniformly. OT effects differed between CBE- and Cas9-edited cells and across the three cell

types, supporting the hypothesis that genotoxicity is both platform and cell type specific. Of particular note, CBE OT activity was detected at twice the number of OT sites compared to Cas9 treatment, and three times the number of translocation sites were identified in CBE- compared to Cas9-edited cells. In addition, longitudinal studies showed that chromosomal rearrangements were more stable in T cells, whereas they rapidly disappeared in HSCs. Consistent with the fact that the frequencies of mutated OT alleles remained stable over time in vitro and in vivo, secondary transplantation of CBE-modified HSCs into 12 mice did not result in graft-related malignant transformation. In conclusion, our results demonstrate successful base editing to reverse the clinical phenotype in a preclinical FHL3 mouse model, but also reveal platform and cell type-specific genotoxicity, highlighting the need for cell type-specific safety studies to properly assess the risk-benefit ratio of these novel technologies.

OR058

Efficient long range gene editing and enrichment of lymphocytes and hematopoietic stem and progenitor cells with the desired editing outcome

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Long range gene editing by homology directed repair (HDR) of primary cells is constrained by efficiency and can result in heterogeneous editing byproducts, including long-range deletions and on-target rearrangements. To address these limitations we first developed droplet digital PCR (ddPCR) and long-range sequencing assays for the quantitative characterization of on-target rearrangements, revealing that on-target potentially adverse outcomes are very heterogeneous and more common than nuclease off-target effects detected by commonly used technologies. Hereby we show that true HDR edited lymphocytes can be enriched with a high degree of purity using a clinical compatible selector functionally coupled to correct editing.

We leveraged on this concept to develop a novel enrichment strategy for HDR-edited haematopoietic stem and progenitor cells (HSPCs) called Selection by Means of Artificial Transactivators (SMArT). In SMArT, the HDR template comprises a selector gene, whose transient expression is conditional to precise on-target integration and delivery of an artificial transactivator (Art) recognizing the genomic region flanking the integration site, outside the donor template cassette. Basal expression of the selector was nearly undetectable, given the presence of only a minimal promoter in the template and was induced to actionable level upon transient mRNA delivery of an ArT. This strategy enriched the HSPC product to >80% HDR-edited alleles. PCR drop-off assays on hundreds of single colonies derived from sorted and bulk edited HSPCs confirmed that >95% of the clonogenic, enriched HSPCs carried the intended HDR edit. Moreover, SMArT efficiently purged out HSPC clones bearing on-target long-range deletions from the positive fraction, alleviating the safety concerns related to genotoxic byproducts of editing.

Transactivation efficiency, however, was significantly reduced in the most primitive HSPCs, possibly due to lower permissiveness of this subset to ArT delivery and/or transcriptional

activation of the edited locus. We hypothesized that this challenge could be addressed by tailoring culture conditions, and developed a new culture and manipulation protocol resulting in significantly higher editing efficiency (»2-4x) upon xenotransplantation with conserved engraftment potential. Moreover, we optimized the selection step, increasing the yield by »2 fold. Xenotransplantation of SMArT-enriched HSPCs resulted in an increase of bona-fide HDR editing from 40% to >99%; correspondingly we observed only a mild decrease of human chimerism compared to a control group receiving the same number of bulk unselected primitive HSPCs. As expected by design, selector expression was no longer detectable in the xenografts. We anticipate that SMArT, when combined with the harvest of large numbers of HSPCs, optimized culture conditions, and possibly the expansion of HDR edited cells may overcome current limitations and concerns of HDR editing, facilitating its clinical translation.

OR059

Chromosomal aberrations, NHEJ scarless repair, recurrent nuclease cleavage and DSB half-life; all at once

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Recent advances in designer DNA editors, including programmable nucleases, PASTE, Prime, and base editors, have demonstrated significant therapeutic potential for treating genetic disorders. However, a comprehensive understanding of their cellular activities is crucial for enhancing editing efficiency and ensuring safe clinical translation of cell and gene therapies. Numerous techniques have emerged to detect or anticipate genotoxic events, reflecting the need for thorough characterization of these editing tools' activities. Nevertheless, these approaches face limitations such as high costs, time-consuming procedures, extensive bioinformatics requirements, and biased evaluations of genetic aberrations. Additionally, the temporal dynamics of DNA editing and subsequent repairs are poorly understood, leading to potential misinterpretations and observational biases.

To address these challenges, we introduce Multipurpose Editing and Genotoxicity Assessment (MEGA), a novel diagnostic toolkit that employs multiplexed digital PCR (dPCR) assays for comprehensive characterization of mutations at both on- and off-target sites induced by gene editing tools. MEGA provides valuable insights into genome integrity and quantifies episomal or integrated DNA donor templates. To demonstrate the broad applicability of MEGA, we employed various designer nucleases (SpCas9, TALEN, Cas12a) to edit clinically relevant primary cells, including human hematopoietic stem and progenitor cells (HSPCs) pre- and post-transplantation in a murine model, induced pluripotent stem cells (iPSCs), and T cells. By quantifying up to ~90% of alleles with unresolved double-strand breaks (DSBs) and other aberrations across various therapeutic gene targets, we exposed biases in commonly used mutation-screening assays.

MEGA's unparalleled ability to assess DNA status post-editing revealed novel insights into the effects of DNA repair inhibitors, designer nuclease activity, and DNA repair mechanisms in primary cells. This includes the elusive yet prevalent precise DSB repair via non-homologous end joining (NHEJ) and subsequent recurrent nuclease cleavage events. This technology also allowed

us to measure mutation probabilities of DSB resolution with unprecedented accuracy and expose the kinetics associated with their resolution by DNA repair pathways.

MEGA addresses critical gaps in the genetic engineering analysis toolkit by providing a rapid, accessible, and specific overview of genome integrity post-gene editing, applicable to clinically relevant samples. Furthermore, by leveraging this novel technique, we elucidated fundamental biological concepts underlying DNA repair, paving the way for extensive mechanistic studies aimed at improving designer editor activity and gene therapy safety.

In conclusion, MEGA stands as a transformative tool for the precise characterization of gene editing outcomes, ensuring safer and more efficient clinical applications of cell and gene therapies. By overcoming the limitations of existing techniques, MEGA enhances our understanding of genome integrity and repair mechanisms, thereby contributing to the advancement of therapeutic gene editing.

OR060

Towards safer engineering of T cells for cancer immunotherapy by polyfunctional editing

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Gene editing of T cells for cancer immunotherapy employs cutting-edge technologies, like CRISPR-Cas9, to enhance the effectiveness of T-cell therapies. These technologies allow precise genetic modifications of T cells, such as targeted expression of Chimeric Antigen Receptors (CARs) or transgenic T-Cell Receptors (TCRs) and genetic inactivation of key genes to improve tumour antigen recognition and enhance T cell function. Application of these approaches has enabled the generation of tumour-specific T cells that are less susceptible to immune inhibitory and suppressive stimuli originating from the tumour microenvironment, potentially improving the efficacy of adoptive T-cell therapy in treating various malignancies. Despite these promising results, multiplex gene editing may lead to several DNA double-strand breaks (DSBs), jeopardizing the genome integrity of treated cells and, hence, the safety of these treatments. To solve this issue, we have developed a novel CRISPR-Cas9–based polyfunctional platform that couples gene and epigenetic editing for efficient, safe, and simultaneous engineering of T cells. We initially established epigenetic editing procedures based on the transient delivery of Engineered Transcriptional Repressors (ETRs) equipped with catalytically deactivated Cas9 in healthy donor-derived primary human T cells. Upon optimization and screening of different ETR designs, we reproducibly obtained robust (up to 90%) and long-term stable (up to 30 days) silencing of three therapeutically relevant genes in T cells. According to the mechanism of action of the ETR technology, the promoters of the ETR-targeted genes were hypermethylated at CpG dinucleotides in epi-silenced cells *vs*. untreated controls. In contrast to Cas9-edited cells, no reciprocal chromosomal translocations were observed among the epi-silenced genes. Furthermore, epigenetically edited CAR-T cells showed sustained gene repression even after recognition and complete eradication of cancer cells *in vitro*. We then designed a polyfunctional editor (poly-editor) endowing the ETR with a catalytically active Cas9 and coupled this design with gRNAs of different lengths to simultaneously program orthogonal edits in T cells. Application of this polyfunctional editing platform in human primary T cells resulted in ≥40% of cells

harbouring durable (≥30 days) epigenetic silencing of multiple genes and targeted integration of a NY-ESO–specific TCR into the *TRAC* locus. Importantly, molecular analyses of poly-edited T cells showed no signs of reciprocal chromosomal translocations among the edited loci. Moreover, when challenged with cancer cells *in vitro*, poly-edited T cells retained their killing activity. We are currently assessing the genome-wide specificity of this platform and expanding the functional characterization of multiplex and poly-edited T cells. Overall, we presented here a novel polyfunctional editing platform, the application of which may allow for safer orthogonal engineering of T cells for cancer immunotherapy.

OR061

Enhancing the potency of *in vivo* lentiviral gene therapy to hepatocytes for hemophilia

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Lentiviral vectors (LV) are attractive vehicles for *in vivo* liver-directed gene therapy, representing a complementary strategy to adeno-associated viral (AAV) vectors. In contrast to AAV, LV integrate into the genome of target cells, potentially ensuring transgene maintenance during liver growth. Moreover, pre-existing immunity against LV components is rare in humans. We have previously shown stable gene transfer to the liver in mice, dogs, and non-human primates by LV administration. However, high quantities of highly purified LV are required for clinical translation. Increasing the potency of gene transfer remains a crucial goal in gene therapy since lowering the dose required to achieve therapeutic efficacy would result in a reduction of dose-dependent toxicities and immunogenicity, besides reducing the costs associated with manufacturing. We focused on LV-mediated *in vivo* gene transfer to hepatocytes of coagulation factor VIII (FVIII) and coagulation factor IX (FIX), the genes mutated in hemophilia A and B, respectively. We tested strategies to increase corrected liver mass *a posteriori* (endowing the corrected hepatocytes with a selective advantage over the non-corrected ones, as previously shown) or *a priori*. To increase potency *a priori*, we evaluated several procedures (fasting, inhibition of interferon, IFN, or proteasome pathways) to implement before LV administration. Specifically, a single intravenous injection of an anti-IFN receptor (IFNAR) antibody (Ab) or the proteasome inhibitor Bortezomib, in adult mice, before LV increased the circulating amounts of coagulation FVIII or FIX of approximately 5-fold. Moreover, fasting adult mice for 24 hours before LV increased transgene output up to 7-fold. We then tested the identified transduction enhancers in combination. While we did not observe an additive effect by combining anti-IFNAR Ab and Bortezomib, we achieved higher transgene output when fasting and anti-IFNAR Ab or fasting and Bortezomib were combined, with the latter combo resulting in approximately 13-fold higher circulating FIX amounts. These findings shed light on molecular pathways that can be targeted to enhance the potency of LV-mediated *in vivo* hepatocyte gene transfer. To increase the potency *a posteriori*, we provided, together with the coagulation FIX or FVIII transgene, a short hairpin RNA downregulating Cypor, the redox partner of all microsomal cytochromes P450s (CYP450s). Since acetaminophen is partially metabolized by CYP450s generating a toxic metabolite, the transduced hepatocytes become resistant to toxic doses of the drug, replacing the damaged ones over time. We confirmed and expanded the feasibility of this strategy, applying it to hemophilia A and B, treating the mice at different ages. We achieved positive selection of transduced hepatocytes,

turning FVIII and FIX transgenes amounts from sub-therapeutic to fully therapeutic. Notwithstanding the clonal expansions, we did not detect any enrichment of LV integrations near oncogenes or tumor suppressor genes in the mice that underwent the selection procedure, suggesting that hepatocyte expansions were not driven by LV integrations, but due to the selective advantage of LV transduced hepatocytes. Here we successfully enhanced the potency of *in vivo* LV-mediated hepatocyte gene transfer, thus paving the way to expanding its *in vivo* applicability and easing its clinical translation.

OR062

Development of a non-viral genetic medicine for Hemophilia A by targeted transcutaneous ultrasound-mediated gene delivery

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The recent approval of a viral vector-mediated gene therapy for Hemophilia A (HemA) provides the potential for a once-a-lifetime treatment option. However, several limitations including high product cost, preexisting immunity to viral vectors, lack of durable FVIII expression, and lack of redosing capability suggest the need to develop alternative non-viral HemA gene therapy treatments to overcome these major limitations. Transcutaneous ultrasound-mediated gene delivery (UMGD) is a non-invasive *in vivo* gene delivery approach that may overcome the key delivery challenges facing genetic medicines.

To evaluate the potential of UMGD for safe and efficient gene delivery, we developed a technology platform utilizing novel acoustic energy profiles and FDA-approved ultrasound components to deliver next-generation genetic payloads to the liver. The delivery process involves intravenous co-administration of DNA payloads and ultrasound contrast agents (microbubbles), coupled with targeted application of externally applied ultrasound energy to guide the DNA into specific tissues. Rapid, durable, re-dosable, and titratable transgene expression was observed upon UMGD-mediated DNA payload delivery to the liver. Durable transgene expression was observed for over one year following a single UMGD treatment. Utilizing RNAscope and snRNA-seq analysis, biodistribution of transgene delivery was evaluated in mouse liver confirming widespread transgene expression in clinically relevant hepatic cell types including hepatocytes and liver sinusoidal endothelial cells (LSECs).

Using genetic engineering and screening of next generation DNA payload formats, we developed an optimized episomal DNA payload expressing an oversized FVIII transgene (FVIII-ST). The FVIII-ST outperformed the clinically validated FVIII-V3 yielding up to 5-fold higher human FVIII expression and secretion *in vitro* and *in vivo*. By optimizing the acoustic energy profiles, we have successfully delivered the FVIII-ST payload to the liver of normal and HemA mice and reached durable therapeutic levels of FVIII in mouse plasma without triggering liver toxicity. Repeated UMGD treatments led to a significant increase in transgenic FVIII levels in plasma up to normal levels, suggesting a redosing treatment regimen is feasible for elevating or maintaining a normal physiological level of circulating exogenous FVIII.

The favorable efficacy and safety results achieved using a novel FVIII-ST payload and UMGD delivery platform support further translation of this approach into clinical development for the treatment of Hemophilia A.

OR063

Stable factor IX expression and sustained reductions in factor IX use 7 years after gene therapy with AMT-060 in adults with haemophilia B

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AMT-060 is an adeno-associated virus serotype 5 (AAV5) vector encoding a codon-optimised wild-type human factor IX (FIX) gene, driven by a liver-specific promoter. AMT-060 has an identical vector sequence to etranacogene dezaparvovec, without the activity-enhancing two nucleotide change in the human FIX coding sequence of the Padua FIX variant. The Phase I/II study included 10 patients with haemophilia B (FIX activity ≤2 IU/dL) who received a single intravenous infusion of AMT-060 (5×10^{12} gc/kg [Cohort 1; n=5] or 2×10^{13} gc/kg [Cohort 2; n=5]). Nine out of ten patients were prophylaxis-free after AMT-060 administration. Using the one-stage activated partial thromboplastin time (aPTT) assay, mean FIX activity as reported initially was 4.4 IU/dL at 52 weeks in Cohort 1 and 6.9 IU/dL at 26 weeks in Cohort 2.

Patients who successfully completed all assessments during 5 years of follow-up were enrolled in the open-label, Phase I/IIb extension study (NCT05360706). Here, we report the second year of follow-up in the extension study; representing 7 years after AMT-060 administration.

Four of five patients from Cohort 1 (including one patient who remained on FIX prophylaxis) and all five patients from Cohort 2 enrolled in the extension study.

FIX activity remained stable at Year 7; ranging from 3.6–6.9 IU/dL in Cohort 1 and 3.7–8.6 IU/dL in Cohort 2 (one-stage aPTT assay). Mean (SD) and median FIX activity were 5.70 IU/dL (1.83) and 6.60 IU/dL in Cohort 1 (n=3, excludes patient who remain on prophylaxis), and 6.68 IU/dL (1.93) and 7.40 IU/dL in Cohort 2 (n=5), respectively.

Mean (SD, n) annualised bleeding rate (ABR, excluding the patient who remained on FIX prophylaxis) for Year 7 was 0.79 (1.37, n=3) and 0.42 (0.58, n=5) for Cohort 1 and 2, respectively, while mean (SD, n) spontaneous bleeding rate (AsBR) was 0.0 (0.0, n=3) and 0.0 (0.0, n=5). In Cohort 1, one prophylaxis-free patient experienced 2 traumatic bleeds, while the patient who remained on prophylaxis experienced 4 traumatic bleeds. In Cohort 2, two patients experienced 1 traumatic bleed each in Year 7.

Mean (SD) annualised FIX consumption during Year 7 (excluding surgeries and the patient who remained on FIX prophylaxis) was 0.0 (0.0) IU/year (or 0.0 [0.0] IU/kg/year) in Cohort 1 (n=3) and 1355.15 (1874.723) IU/year (or 15.80 [21.70] IU/kg/year) in Cohort 2 (n=5).

No new safety events were identified during Year 7, and no patient returned to continuous FIX prophylaxis.

Gene therapies for haemophilia A and B, including etranacogene dezaparvovec, were recently authorised in Europe. Durability of factor expression is a key consideration in the decision-making process for patients and physicians. This 7-year follow-up after AMT-060 administration confirms the safety, durability and stability of FIX expression after AAV-based gene therapy reported previously.

OR064

Haematopoietic stem cell gene therapy as a treatment for NOD2-deficient severe Crohn's Disease

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Pathogenic variants of the *NOD2* (nucleotide-binding oligomerization domain containing protein 2) gene demonstrate the strongest genetic association to Crohn's inflammatory bowel disease (CD), with mounting evidence linking NOD2 deficiency with poor clinical outcome. CD patients with homozygous or compound heterozygous NOD2 pathogenic mutations, present an aggressive, fistulizing and fibrostenotic disease, requiring multiple surgical resections. NOD2 deficiency is implicated to drive CD pathogenesis through failure of gut innate immunity to detect and resolve bacterial infections and by loss of tissue homeostasis within the intestinal microenvironment. Here we present preclinical data on OTL-104, an autologous *ex vivo* haematopoietic stem cell gene therapy (HSC-GT) approach, which aims to stably restore NOD2 expression in gut resident macrophages. We demonstrate the safety and efficacy of an HSC-GT approach to correct immune dysfunction due to NOD2-deficiency, by harnessing the natural capacity of gene-modified HSCs to differentiate into monocytes/macrophages that continuously migrate to and repopulate the intestine, and restore NOD2-dependent functional immunity.

We used *in vitro* and *in vivo* models of NOD2 deficiency to demonstrate the mechanism of action and the efficacy of OTL-104 autologous HSC-GT. $NOD2^{KO}$ human myeloid cells differentiated *in vitro* from CRISPR-generated $NOD2^{KO}$ $CD34^{+}$ HSCs are unable to mount a proinflammatory cytokine response to stimulation with muramyl dipeptide (MDP, NOD2 agonist). Similarly, myeloid cells differentiated from $CD34^{+}$ cells obtained from peripheral blood of genetically characterized NOD2-deficient CD patients, are also refractory to MDP stimulation and unable to generate a normal cytokine response profile. In both NOD2 deficient $CD34^{+}$ derived monocyte models, transduction with a lentiviral vector (LV) expressing NOD2 fully restores NOD2-dependent cytokine and chemokine responses, resulting in a functional immune response profile that is comparable to monocytes derived from $CD34^{+}$ cells from NOD2 wild-type healthy donors. Transplantation of lineage negative (Lin^{-}) haematopoietic stem/progenitor cells (HSPCs) transduced with the OTL-104 LV in $NOD2^{KO}$ mice was used as an *in vivo* model of gene therapy for CD. Compared to WT mice, $NOD2^{KO}$ mice fail to release systemic inflammatory mediators and recruit myeloid cells in response to MDP administration. Transplantation of transduced Lin^{-} HSPCs restores MDP-induced systemic release of IL-6 and CXCL1 as well as mobilization of myeloid cells, demonstrating correction of defective NOD2-dependent innate immune cell activity by HSC-GT. Key to our therapeutic approach, histopathological analysis of

intestinal *lamina propria* from transplanted mice shows a normal biodistribution and physiological NOD2 gene expression is achieved in tissue resident cells. Importantly, we also demonstrate complete restoration of an intestinal tissue response to *in vivo* administration of MDP in HSC-GT treated $NOD2^{KO}$ mice. Gene expression ontology analysis of gut tissue from transplanted mice confirms that NOD2-dependent innate immune activation, intestinal tissue remodeling and immune homeostasis signaling is fully corrected to levels observed in WT mice.

These results confirm the impact of NOD2 deficiency in primary immune activation which drives disease induction and pathology in Crohn's Disease and demonstrates the therapeutic potential of OTL-104 HSC-GT to achieve long-term correction of severe disease in NOD2-CD.

OR065

mRNA therapy for acute myocardial infarction

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There is a significant need to develop innovative therapies for myocardial infarction, aimed to prevent cardiomyocyte death in the first days after the acute event, extend the period when revascularisation remains effective, and protect the heart from reperfusion damage. With a systematic approach based on the in vivo functional selection (FunSel) of AAV-expressed proteins, we have previously identified three novel molecules (Chrdl1, Fam3c and Fam3b) that exhibit the most potent cardioprotective properties out of 1200 screened factors. Here we describe an effective method for the in vivo administration of the mRNA encoding these proteins. We developed liver-targeted lipid nanoparticles (LNPs) for the transient expression of the cardioprotective proteins. We screened several LNPs generated with the SNALP technology for effective liver transfection. Eventually, we identified a specific formulation that permits broad hepatocyte transfection with mRNA coding for the cardioprotective protein and sustains protein secretion into the circulation without eliciting apparent innate immune and inflammatory response. Using this LNP formulation, we administered the modified cardioprotective protein mRNA intravenously shortly after myocardial infarction in mice. Protein secreted into the blood started to be detected as early as 4 hours after administration, reaching the highest concentration after 16 hours, and started to decline after 72 hours. A single LNP/mRNA administration was sufficient to protect the heart against cardiac damage (as assessed by the measurement of infarct size 30 days after infarction by heart morphometric analysis), sustain cardiac function and prevent adverse cardiac remodelling (by echocardiography). These results pave the way to the development of a novel generation of therapeutics for acute applications, based on the systemic administration of liver targeted mRNAs that produce proteins secreted into the systemic circulation to act in the heart, or in other distant organs.

OR066

Ten-year efficacy of gene therapy in a canine model of X-linked myotubular myopathy

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X-linked myotubular myopathy (XLMTM) is a rare congenital myopathy characterized by severe muscle weakness, respiratory insufficiency, and premature death. A p.N155K missense mutation in the *MTM1* gene in dogs also leads to a reduction in life expectancy and a progressive generalized myopathy, with muscle fibers presenting the histopathological hallmarks of the disease. Previous results demonstrated the therapeutic effect of an AAV8 vector carrying the *MTM1* gene under the control of a desmin promoter after a single systemic injection. The treatment prolonged the survival of XLMTM dogs, restored muscle strength, and improved respiratory, gait and neurological functions. Currently, several clinical trials, such as ASPIRO for XLMTM, use AAV vectors to treat neuromuscular diseases. The first patients were included approximately 5 to 7 years ago. However, critical questions remain about the persistence of transduced myofibers for several years while undergoing normal turnover and the persistence of transgene expression at therapeutic levels with time. Here, we followed over a 10-year period, two XLMTM dogs treated at 10 weeks of age with 2e14 vg/kg. Annual biopsies from several muscles were analyzed for vector copy number, transgene mRNA level and protein expression. Transduced myofiber levels remained stable in the biceps brachii, vastus lateralis and cranial tibialis. Canine *MTM1* mRNA expression in muscles persisted over time, above the endogenous levels. Immunoblots from treated dogs revealed protein amounts similar to or higher than those in healthy dogs, with a broad distribution in hindlimb and forelimb muscles as well as the diaphragm 10 years after injection. Neurological assessments, gait analysis, respiratory function and bilateral hindlimb force testing were performed yearly. Results were similar to those previously published from years 1 to 4, and indistinguishable from one littermate control. Muscle histology at 10 years post-dosing displayed nonspecific myopathic features, but none were characteristic of XLMTM. Together, our findings indicate that the efficacy of AAV8 gene therapy for a congenital myopathy persists for at least one decade in a large animal model through stable expression of the therapeutic protein.

OR067

NVC-001 – an AAV gene therapy for LMNA-related dilated cardiomyopathy

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LMNA-related dilated cardiomyopathy (LMNA-related DCM) is caused by mutations in the LMNA gene and comprises 5-6% of genetic DCM cases with approximately 50,000 patients in USA alone. The natural course of LMNA-related DCM is aggressive, often leading to premature death or heart transplant. Patients typically initially present with cardiac conduction system disease in the third and fourth decades of life that progresses to ventricular arrhythmias, decline in left ventricular ejection fraction (LVEF) and myocardial fibrosis, eventually deteriorating rapidly to end-stage heart failure. Most DCM-related LMNA mutations are autosomal dominant and gain-of-function, precluding conventional gene replacement as a treatment option. There are no specific therapies for LMNA-related DCM.

To address this substantial unmet need, NVC-001 is currently under preclinical development as a potential curative treatment for LMNA-related DCM patients. NVC-001 is an adeno-associated virus (AAV9) vector that drives cardiac-specific expression of a dominant-negative form of the naturally occurring human SUN1 protein (AAV9-dnSUN1). A 26-week safety study performed in non-human primates with a single intravenous dose of NVC-001 confirmed highly specific transgene mRNA and protein expression in the heart compared with other organs. Clinical pathology and histopathological analyses also demonstrated that NVC-001 is well-tolerated. Proof-of-concept studies with NVC-001 have been conducted in a well-characterized tamoxifen-inducible, cardiomyocyte-specific Lmna conditional knockout (Lmna-DCM) mouse model. We demonstrate that a single dose of NVC-001 in a treatment setting confers significant survival benefit in disease-bearing Lmna-DCM animals, accompanied by improvements in cardiac function phenotypes as measured by both echocardiography and electrocardiography across a range of clinically relevant doses. Furthermore, treatment with NVC-001 rescues the arrhythmic phenotype observed in human induced pluripotent stem cell-derived cardiomyocytes harboring the pathogenic LMNA-N195K point mutation that is associated with DCM in humans, supporting the translatability of NVC-001 as a treatment for LMNA-related DCM in human patients.

In summary, NVC-001 holds significant promise as a potential treatment for LMNA-related DCM.

OR068

AAV delivery of wild type TMEM43 as a novel therapeutic approach for arrhythmogenic right ventricular cardiomyopathy type 5

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Arrhythmogenic right ventricular cardiomyopathy type 5 (ARVC5) is the most aggressive type of ARVC, caused by a fully penetrant missense mutation (p.S358L) in TMEM43. Pathologically, the disease is characterized by dilation of the cardiac chambers and fibro-fatty replacement of the myocardium, which results in heart failure and sudden cardiac death. Current therapeutic options are limited and no specific therapies targeting the primary cause of the disease have been proposed.

TMEM43 function and its role in ARVC5 pathogenesis are virtually unknown. We have observed that the p.S358L variant in TMEM43 does not affect its self-binding ability, suggesting that the overexpression of WT-TMEM43 could quench the deleterious dominant effects of the mutant protein by forming a WT-S358L heterodimer. In a first proof-of-concept approach, we generated a double transgenic mouse that overexpresses both WT and mutant TMEM43 in cardiomyocytes. In a second, highly-translational approach, we used self-complementary myotropic AAVs to express WT and mutant human TMEM43 under cTnT promoter (AAV-WT and AAV-MUT, respectively). Mice were followed for 24 weeks and cardiac function was evaluated using echocardiography and electrocardiography (ECG).

Both the transgenic mutant model and the mice infected with AAV-MUT recapitulated the main characteristics of the human disease, including cardiomyocyte death, ventricular dysfunction and ECG abnormalities. Double transgenic mice showed delayed ARVC5 onset, significantly improving survival compared to transgenic mutant mice. Similarly, co-administration of AAVs bearing WT and S358L-TMEM43 resulted in increased ejection fraction and QRS amplitude, reduced cardiac troponin levels in serum and downregulation of fibrosis-related genes, compared to AAV-MUT injected mice.

The data presented here underscore the therapeutic potential of TMEM43 overexpression in ARVC5, with no apparent detrimental effects of TMEM43 overexpression in the heart under healthy conditions. Importantly, AAV-mediated delivery of WT-TMEM43 prevents from most of ARVC5 features including premature death, and offers a promising and specific therapy for patients suffering from this highly lethal disease.

OR069

Results from the CAN*aspire* Gene Therapy Trial for Canavan Disease: Safety, Biomarker, Imaging, and Clinical Outcome Data from the Completed Low-dose Cohort

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Canavan disease (CD) is an early-onset fatal spongiform leukoencephalopathy caused by autosomal recessive loss-of-function mutations in the *ASPA* gene. Deficient ASPA enzymatic activity leads to elevated levels of its substrate, N-acetylaspartate (NAA), and profoundly impaired psychomotor development, with typically affected patients failing to gain even the most basic milestones. CAN*aspire* (NCT04998396) is an ongoing first-in-human open-label study evaluating the safety, pharmacodynamic, and clinical activity of BBP-812, a recombinant AAV9 h*ASPA* vector administered systemically for the treatment of CD.

Participants receive one intravenous infusion of BBP-812 along with glucocorticoid immune prophylaxis for 3 months followed by a gradual taper. Safety is assessed by standard laboratory, physical and neurological assessments. NAA is quantified in urine and CSF by GC-MS and in brain by MRS. Effects on white matter pathology and brain volumes are evaluated by MRI. Clinical outcome measures include the disease-specific CD Rating Score (CDRS) that ranks the severity of 11 characteristic neurological and developmental features of CD, as well as performance-based and parent-reported pediatric motor and developmental scales. CAN*inform* natural history data (NCT04126005) are used as a comparator for NAA and clinical/functional assessments.

To date, nine participants have received BBP-812: eight at 1.32 x 10^14 vg/kg (low-dose cohort) and one at 2.6 x 10^14 vg/kg. Mean age at dosing was 18.2 months (median 18.0 months) with an age range of 9.6 to 29.2 months. Most adverse events (AEs) have been mild or moderate and unrelated to BBP-812. Related AEs have generally been consistent with other systemic AAV9 gene therapy products, including transient self-limited thrombocytopenia as well as elevated liver enzymes that have been managed with a temporary increase in glucocorticoid dosing.

All participants have demonstrated rapid, robust and durable NAA decreases in urine, CSF and brain over 0.3 to 2.6 years of follow-up to date (mean 1.4 years, median 1.2 years), with good correlation between urine and brain NAA concentrations (Pearson r = 0.736, p-value = 0.024). Post BBP-812 dosing, urine NAA has fallen from baseline levels characteristic of classic, severe CD (above 1,000 mmol/mol creatinine) to levels associated with milder CD phenotypes (below 750 mmol/mol creatinine), as reported in the literature and observed in the CAN*inform* natural history study. MRI has shown resolution of white matter swelling and evidence of new myelination in the brainstem and cerebellar peduncles. Participants have generally stabilized existing motor skills or gained new skills, in some cases beyond the typical function of CD patients. Aggregate clinical data show developmental and functional gains after BBP-812 administration compared to natural history.

The low-dose cohort has been completed and participants entering the study are currently receiving BBP-812 at a dose level of 2.6E14 vg/kg. Preliminary data from the higher-dose group will be included in this presentation as available.

OR070

Non-clinical and early clinical development of PBFT02, an AAV gene therapy for FTD with *GRN* mutations (FTD-*GRN*)

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Frontotemporal dementia (FTD) is a neurodegenerative clinical syndrome characterized by a spectrum of disturbances across behavior, executive function, and language domains. Approximately 25-30% of FTD is caused by autosomal dominant mutations, usually in one of three genes, *C9orf72*, *GRN*, or *MAPT*. In FTD-*GRN*, *GRN* mutations result in a progranulin (PGRN) haploinsufficiency leading to neurodegeneration, likely related to lysosomal dysfunction and inflammation. There are currently no disease-modifying treatments approved for FTD.

PBFT02 is a recombinant adeno-associated virus serotype 1 (AAV1) vector that expresses human progranulin (hPGRN) protein in neurons, glia, and ependymal cells. Since PGRN is a secreted protein, PBFT02 expression in transduced cells can additionally impact surrounding cells, leading to the phenomenon of cross-correction. PBFT02 was selected as a development candidate based on proof of concept studies showing efficacy of cerebrospinal fluid (CSF)-delivered AAVhu68.hPGRN in *GRN* deficient mice, and on superior hPGRN expression in non-human primates (NHPs) following administration of AAV1.hPGRN compared to other AAV capsids. Here we report the non-clinical characterization of PBFT02. Following intra-CSF administration of PBFT02 to *GRN* knockout mice, increased CNS expression of hPGRN was associated with improvements in lysosomal function and reduced neuroinflammation. Intra-CSF delivery to NHPs, via intra-cisterna magna (ICM) administration, resulted in extensive and robust expression of hPGRN throughout the brain and spinal cord of non-human primates, and PBFT02 was safe and well tolerated over the dose range examined.

PBFT02 is currently being studied in a first-in-human dose escalation clinical trial, upliFT-D, in FTD-*GRN* individuals (NCT04747431 [ClinicalTrials.gov]). The primary objective is safety and tolerability, and secondary objectives include biomarkers of target engagement (PGRN expression), biological activity, and disease progression. The 2-year trial will be followed by a 3-year extension for safety and durability of effect. Participants receive a one-time administration of PBFT02 directly to the CSF via a single CT-guided ICM administration. As of June 2024, all 5 subjects in Cohort 1 have received PBFT02 at a dose of 4.5×10^{13} genome copies. PBFT02 was generally well-tolerated with a steroid immunosuppression regimen of 1 g IV methylprednisolone on study days 1 to 3, followed by 60 mg oral prednisone on days 4 to 60. At baseline, levels of CSF and plasma PGRN were below levels in healthy adult controls, as expected in FTD-*GRN*. Following PBFT02 administration in the initial 3 participants, CSF PGRN levels increased to 2.2 to 3.6x higher than the mean of healthy adult controls by day 30, and increased further to 4.6 to 5.7x above healthy adult controls in the first 2 participants followed out to 6 months post-dose.

In summary, the favorable non-clinical profile of PBFT02 led to the initiation of a Phase 1/2 clinical study in FTD-*GRN* individuals. Interim safety and biomarker data from the upliFT-D trial provides initial evidence that PBFT02 has potential as a one-time therapy for FTD-*GRN* and supports its further clinical development.

OR071

Twenty-year Survival Analysis of CNS AAV2-mediated Gene Therapy for CLN2 Disease

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CLN2 disease (late infantile neuronal ceroid lipofuscinosis) is an autosomal recessive, neurodegenerative lysosomal storage disease affecting the central nervous system (CNS), with death typically at ages 8-12. Twenty years ago, we initiated a phase I clinical trial to treat CLN2 CNS disease with administration to the central nervous system of AAV2hCLN2, a serotype 2 AAV vector delivered to 12 sites in the brain through 6 burr holes [total dose 2.5 $x10^{12}$ particle units (NCT00151216)]. Ten children were treated at an average age of 6.0±0.7 years. The average severity on the modified Hamburg Clinical score was 4.2±0.4 units. One died 49 days post-therapy, with persistent seizures. Other adverse events were limited in duration and severity, and none were attributed to the vector. Of the 9 other treated children, at one-year post-therapy, there was a significant improvement in the modified Hamburg Clinical score ($p<0.01$, Worgall, S et al, Human Gene Therapy, 2008). The focus of the present study is an analysis of survival of these 9 treated children, 20 years from the time the first patient was treated. On the average, despite the significant clinical improvement at 1 year, the median survival of the 9 treated children was 10.1±2.5 years, similar to that of 2 untreated natural history control groups, Hamburg 9.8±3.7 years, $p>0.2$, Weill Cornell 11.1±1.1 years, $p>0.2$. However, analysis of the survival of the 9 treated children demonstrated there were 2 survival groups, with a wide disparity in survival. Group 1 (short survivors, n=6) had a median survival to 8.8±0.4 years of age, 4.1±0.5 years post-therapy. Strikingly, group 2 (long survivors, n=3) had a markedly longer median survival to 24.5±2.4 years of age, 14.5±2.8 years post-therapy ($p<0.0001$, survival of group 1 *vs* group 2). Interestingly, assessment of the long survivors demonstrated they were treated at an older age (group 1: 4.5±0.3 yr; group 2: 8.6±0.9 yr) and had more severe disease at time of treatment (Hamburg clinical score group 1: 5±0.3, group 2: 3±0.0, $p<0.02$). Genotype analysis revealed that while the 3 long survivors had one allele (G3356C) identical to that in 3 of the short survivors, the 2nd allele was different. However, this was unlikely to explain the survival difference, as genotype analysis demonstrated that for the group 2 long survivors, both alleles were predicted deleterious by the Combined Annotation Dependent Depletion (CADD) score of 32.8±0.3 (a score of >20 is considered deleterious), no different from that of group 1 short survivors (CADD score 35.0±0.8, $p>0.06$). This study represents one of the longest survival analysis of individuals with hereditary disorders treated with AAV vectors. Survival analysis up to 20 years demonstrates that gene therapy can have variability of therapeutic efficacy based on an underlying genotype, where the genotype on its own has no apparent survival advantage.

OR072

Targeting astrocytes with editing technologies to treat Alexander Disease

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Alexander disease (AxD) is a progressive and fatal leukodystrophy caused by gain-of-function mutations in the glial fibrillary acidic protein (GFAP), which encodes the main intermediate filament of astrocytes. Accumulation of GFAP aggregates in Rosenthal fibers leads to Central Nervous System (CNS) dysfunction with typical pathological traits, including demyelination, neuroinflammation, megalencephaly, seizures and spasticity. No cure is currently available for this fatal neurodegenerative disorder. Here, we aim to develop a novel, single-dose gene editing strategy silencing GFAP expression as a lifetime treatment of AxD.

We performed in vitro screening of single guide RNAs (sgRNAs) targeting the *Gfap* gene and selected the best sgRNA candidate inducing a robust GFAP knock-down, while no gene editing at top off-target loci was evident. To optimize the *in vivo* brain-directed delivery of the *Gfap*-targeting CRISPR system, intracerebroventricular injections in mice defined the optimal AAV serotype, age of treatment and glia-specific promoter to maximize transgene expression in astrocytes of AxD-affected brain regions. A single AAV carrying the *Gfap*-targeting sgRNA and Staphylococcus aureus Cas9 nuclease was administered in neonatal *Gfap*+/R76H AxD mice, resulting in on-target editing in astrocytes (up to 30%), robust GFAP downregulation, reduced astrogliosis and mitigated accumulation of Rosenthal fibers - a hallmark of AxD pathology - in white matter regions. To expand on the potential of gene editing as a treatment for AxD, we optimized allele-specific strategies targeting the hotspot mutations detected in AxD patients. We identified adenine base editors that correct the R76H *Gfap* mutation in vitro with minimal off-target activity, and we are currently optimizing its application *in vivo* in AxD mice.

Overall, these data provide in vivo proof-of-concept of the efficacy of a CRISPR/Cas9 editing approach in ameliorating disease-associated phenotypes. Also, our results pave the way for the broadening of gene editing tools that apply mutation-specific treatment to target the mutated *Gfap* allele in the CNS, explicitly using AAV vectors or, prospectively, non-viral delivery systems. Novel editing platforms for in vivo targeting of CNS astrocytes could benefit AxD and other neurodegenerative disorders characterized by primary astrocyte degeneration or dysfunctional/maladaptive astrogliosis.

OR073

Cancer Vaccines: Anti-tumoral T cell therapy on demand

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Cancer immunotherapies exploit the patient´s own immune system to seek and destroy cancer cells. Such therapies so far unfortunately still can help only a subset of patients. Therefore, immunological approaches hold a great potential for cancer treatment, but need strong and solid optimization and better understating. Among them, immune adjuvants and cancer vaccines showed powerful activity not only in clearing the neoplasia but even in giving a long-lasting immune memory avoiding disease recurrence. Despite its great success and its enormous potential in treating patients, cancer immunotherapy lacks successful responses with a broader efficacy, mostly due to immunosuppression and natural immune regulation against self.

There is now newfound knowledge on how to mitigate suppression with the use of adjuvants for cancer treatment. Among them viruses and bacteria showed a great potential in general activation of the immune machinery. Those immunostimulants are excellent in triggering the immune system to an initial stage but they suffer from lack of specificity for tumor type and miss the long-term efficacy over time to serve as vaccines. Furthermore, the mere activation of the immune system does not suffice to clear out malignancies, for which specialized cancer killer cells are needed. Those cells are generally cytotoxic T cells (CTLs) which upon proper activation they seek and destroy cancer bearing features.

The need for both adjuvant and specificity brought us to generate and investigate potential personalized cancer vaccination technologies like loading oncolytic viruses, other microbes or polymers serving as adjuvant, with specific tumor bearing signatures called Tumor Associate Antigens (TAAs) as targets for cancer killing cells.

The results of our new therapies showed a great potential in fighting tumors not only as a therapy in several mouse models, but by establishing immunological memory, which confers long term protection against the disease relapse. We could further generate a specific repertoire of cytotoxic T cells able to clear and control the tumor growth. From this, the next idea was to bring already approved or soon to be approved existing vaccines to a new stage, turning them into powerful anticancer therapies to develop versatile, personalized, and efficient platforms. We focused on Oncolytic Viruses (OVs), Porous Silicon nanoparticles (PSi), Bacillus Calmette Guerin bacteria (BCG) and Measle Mumps Rubella (MMR) vaccine as immune stimulants.

We monitored the efficacy of such new technologies in vitro with assays directly performed on immunological cells and then in different in vivo murine tumor models to assess the general efficacy in immunocompetent and immune- deficient complex systems. All the technologies were able to induce and potentiate cancer cell killing showing improved efficacy addressing the major drawbacks of immunotherapies. This work shows the possibility of using the immune system as a strong weapon in fighting tumors when properly activated and instructed. Polymer nanoparticle, oncolytic viruses and bacteria are powerful activators of an immune reaction and the physical combination of them with TAAs create a strong tool to act as customizable and personalized vaccines, triggering effector T cells for treatment and long-lasting T cell memory for prevention.

OR075

Epitope editing combined with extended schedule anti-KIT antibody treatment enhances immune-based in vivo selection of multiplex genome-engineered cells

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Hematopoietic stem/progenitor cell (HSPC) transplantation (HSCT) offers curative options for conditions for which the substitution of endogenous hematopoiesis with gene-corrected cells can halt the pathogenic process. Yet, short and long-term effects of genotoxic conditioning remain a barrier to a wider application of HSCT and gene therapies. Whereas monoclonal antibodies (mAbs) targeting HSPCs have been proposed as alternative to chemo/radiotherapy, their pharmacokinetics hamper effective clinical use due to risk of on-target depletion of transplanted HSPCs, leading to increased risk of graft failure or incomplete myeloablation. We previously demonstrated that precise editing of the targeted epitope in HSPCs can endow hematopoietic cells with selective resistance to mAbs or CAR-T cells without affecting stem cell functionality. We identified amino-acid changes in the extracellular domain 4 of KIT that abrogate the binding of a therapeutic mAb (Fab79D) without affecting surface expression, ligand affinity and downstream signaling. We exploited adenine base editing (BE) to efficiently (~80%) introduce these mutations in CD34+ HSPCs with preserved long-term multilineage repopulation. As epitope-editing can be achieved through BE, it is well suited for combination with additional therapeutic genome modifications, such as the disruption of *BCL11A* erythroid enhancer to enforce fetal hemoglobin (HbF) expression, which can provide therapeutic benefit for patients affected by sickle cell disease. We multiplexed KIT and sgRNAs targeting the +55 and +58 BCL11A DNase I hypersensitive sites (DHSs) and obtained efficiencies comparable to single edits, while achieving upregulation of HbF proportional to the number of disrupted WGATAR motifs (up to 80%). Critically, we observed high degree of biallelic co-editing, with 60% HSPCs bearing biallelic KIT edits and at least 3 disrupted WGATAR motifs. By performing a competitive transplant with co-injection of multiplex KIT+BCL11A BE and AAVS1 BE CD34+ cells in NBSGW mice. We observed progressive enrichment of both *KIT* and *BCL11A* edits (~2x) in mice treated with a standard dose-dense Ab regimen when compared to controls. We speculated that optimized Ab schedules with extended exposure to anti-KIT mAb may provide superior results. We performed a second competitive transplant with lentiviral fluorescent marking of the KIT+BCL11A BE (mTagBFP) and AAVS1 BE cells (mNeonGreen), to enable precise tracking by FACS. We treated mice with a dose-dense or extended Ab regimens (6 doses, either every 5 or 10 days). Extended regimens produced markedly improved in vivo selection with near complete replacement (>80%) within progenitor and myeloid/erythroid compartments, which translated to improved HbF increase. The selection was proportional to KIT surface levels and lineage half-life. As epitope editing eliminates limitations due to Ab on-target toxicity, we can envision innovative, non-genotoxic gene therapy transplant regimens that will achieve therapeutic levels of corrected HSPCs with substantial advantages over alternatives in terms of safety and tolerability.

OR076

RNA Gene Writers drive therapeutically relevant *in vivo* correction of monogenic disease mutations in the liver and hematopoietic stem cells

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Monogenic diseases are caused by mutations in a single gene, and although most monogenic diseases are rare, together they affect millions of patients worldwide. RNA Gene Writers are designed to introduce a broad range of alterations to the genome, from writing in a transgene, inserting an exon, to introducing single nucleotide changes, providing an attractive toolkit for the treatment of these diseases. RNA Gene Writers leverage target-primed reverse transcription (TPRT), a mechanism that evolved from retrotransposons to introduce DNA sequences into the genome using an RNA template. RNA Gene Writers are designed to be delivered as all-RNA compositions, which facilitate *in vivo* delivery by lipid nanoparticles (LNPs).

Here we explored the application of RNA Gene Writers to correct mutations in the liver and hematopoietic stem cells (HSCs), which lead to various monogenic diseases including alpha-1 antitrypsin deficiency (AATD) and sickle cell disease (SCD), with the aim of achieving therapeutically relevant levels of correction *in vivo*. We developed an RNA Gene Writer designed to correct the E342K mutation (PiZ allele) in the *SERPINA1* gene responsible for AATD. LNP--RNA delivery of an RNA Gene Writer corrected this mutation in an existing transgenic mouse model with high copy numbers of the mutation (*NSG-PiZ*), where we achieved an average of 55% alleles and 93% of mRNA transcripts corrected after a single intravenous administration. We also observed significant increases in serum AAT levels and reductions in protein aggregates in the liver. Separately, we engineered a humanized mouse model with only two PiZ alleles to better approximate patients with the homozygous PiZZ genotype. LNP-RNA delivery of an RNA Gene Writer achieved an average of 49% correction after a single low dose (0.1 mpk) in this diploid mouse model with a corresponding increase in serum AAT. An initial study in NHPs demonstrated RNA Gene Writers achieved an estimated 56% rewriting efficiency at *SERPINA1* in hepatocytes that corresponded to 64% of mRNA transcripts containing the edited sequence.

RNA Gene Writers were also designed to correct the SCD mutation (E6V) to wild-type in the HBB gene. We first modeled the efficiency of HBB editing by introducing the Makassar variant (E6A) in CD34+ human HSCs from healthy donors. We observed an average of 74% correction *in vitro* after optimization of the RNA template, delivery, and RNA Gene Writer expression. We saw high engraftment levels and maintenance of HBB correction in long-term repopulating HSCs and their multi-lineage progeny isolated from mice at 16 weeks post-transplant. Comparable editing levels were also maintained after secondary transplant. Humanized mice receiving an RNA Gene Writer formulated in an LNP showed an average of ~27% and ~44% HBB rewriting *in vivo* in HSCs after the first and second dose, respectively. By combining our RNA Gene Writers and LNP delivery platform, we believe we could potentially correct the SCD mutation to wild-type by targeting HSCs *in vivo*.

Taken together, we demonstrated that our RNA Gene Writers can efficiently correct disease-causing mutations *in vivo* and have the potential to be applied in a range of therapeutic applications.

OR077

LNP delivered Prime Editors restore glycemic control in humanized rodent models of Glycogen Storage Disease Type 1b (GSD1b)

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GSD1b is an autosomal recessive disorder caused by mutations in the *SLC37A4* gene encoding the glucose 6-phosphate translocase (G6PT) which is required for normal glucose-6-phosphate metabolism. Patients exhibit multiple clinical manifestations, including severe fasting hypoglycemia resulting in seizures and cognitive impairment. Clinical management of the disease is inadequate, focusing on mitigating complication severity rather than addressing the underlying cause of the disease. Currently, there are no approved therapies for GSD1b, highlighting the need to develop a therapeutic that provides durable benefit. The most prevalent mutations include p.G339C and p.L348fs, observed in ~50% of patients. A one-time gene editing therapy to correct these causative mutations in hepatocytes would directly address the underlying genetic cause of the disease.

Prime Editing is a next generation gene editing technology that can precisely correct more than 90% of all pathogenic human mutations without the need for double strand breaks (DSBs) and with minimal byproducts at the edit site, minimal off-target activity, and minimal risk of chromosomal alterations or genotoxicity observed with traditional CRISPR-based editing. We have generated LNP-RNA Prime Editor (PE) candidates that are lipid nanoparticles (LNP) formulated with an engineered mRNA that encodes the Prime Editor protein and the Prime Editor guide RNA (pegRNA) with the aim of correcting the mutated *SLC37A4* gene in hepatocytes.

High-throughput screening for pegRNA identified Prime Editors that precisely correct either the p.G339C or p.L348fs mutation. Assessment of LNP-RNA PE candidates in primary mouse hepatocytes, isolated from humanized mice in which the mouse *Slc37a4* gene was replaced with the human gene harboring the targeted mutation, resulted in editing of ≥70% for both mutations. Using Prime Medicine's suite of biochemical and targeted sequencing assays, initial in vitro studies detected no off-target editing in human hepatocytes.

In vivo, intravenous (IV) delivery of the same LNP-RNA PE to humanized mutant mice resulted in precise correction of the p.G339C or p.L348fs mutations in whole liver at an average rate of 52% and 88% in hepatocytes, respectively. In p.L348fs humanized mice, precise correction was associated with correction at the mRNA level, restoration of *SLC37A4* transcript levels and increased protein expression. Homozygous *SLC37A4* p.L348fs and p.G339C mice die perinatally from hypoglycemia and growth retardation. IV delivery of LNP-RNA PE candidates resulted in increases in body weight and improvement in blood glucose and other metabolic parameters to levels approaching those of healthy mice.

In non-human primates, IV delivery of NHP surrogate LNP-RNA PE resulted in >55% editing and undetectable unintended edits in hepatocytes. In addition, the durability of intended edits was confirmed by longitudinally evaluating editing rates in non-human primates at several timepoints following initial delivery of LNP-RNA PE up to 16 weeks, the last time point measured.

These results demonstrate Prime Medicine's LNP-RNA Prime Editors are efficiently and safely delivered to primate liver, resulting in durable, efficient and precise correction of two prevalent mutations and reversing hypoglycemia and growth retardation in humanized mice.

OR078

CRISPR-Cas9 mediated endogenous utrophin upregulation improves Duchenne Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a lethal progressive neuromuscular disorder affecting 1:5000 newborn males. It is caused by mutations in the *DMD* gene, leading to a loss of expression of the dystrophin protein. This induces inflammation, necrosis, and fibrosis, resulting in muscle wasting. There is currently no curative treatment for this devastating disease, which is lethal around the age of 20-30 years old. Several dystrophin-based therapies are currently under study such as exon-skipping, AAV-mediated delivery of mini/micro-dystrophin constructs or CRISPR-mediated editing strategies. However, these strategies propose to restore truncated forms of dystrophin, converting the phenotype to a Becker-like dystrophy, and might induce immune response to the newly expressed dystrophin. In addition, most strategies are mutation-dependent, therefore only subsets of patients could benefit from the treatment. An alternative therapeutic approach, potentially suitable to all DMD patients irrespective of their genetic defect, consists in upregulating utrophin (UTRN), a structural and functional paralogue of dystrophin, able to compensate for the dystrophin deficit. Several studies have shown that its overexpression ameliorates the dystrophic phenotype in mice and dogs with a lower risk of immune response. While a 1.5-fold increase of utrophin results in a therapeutic benefit, a 3-4-fold overexpression prevents the dystrophic pathology. Here, we developed a CRISPR-Cas9-mediated strategy to increase utrophin expression by relieving its repression. Previous work has shown that UTRN is post-transcriptionally downregulated by the Let-7c microRNA (miR) binding its 3' untranslated region (3'UTR). Using a Cas9/gRNA ribonucleoprotein complex we disrupted the binding site (BS) of this miR at the DNA level in human DMD and murine myoblasts to permanently upregulate utrophin. This induced a 3.5- and 3-fold increase of UTRN expression at the mRNA and protein levels respectively. Results were confirmed in three-dimensional human DMD cultures, where Let-7c BS disruption resulted in over 2-fold UTRN upregulation and functional improvements of calcium intake and muscle contraction. In addition, we performed Guide-Seq analysis and didn't observe any major off-target editing for the selected Let-7c targeting gRNA. Finally, we evaluated this strategy in the mdx mouse model of Duchenne. Since miRs are known to be dysregulated in DMD, we first confirmed by qPCR that miR Let-7c expression is not affected in the tibialis anterior, diaphragm and heart of this model compared to the wild-type C57BL10J mice. We then performed intravenous injection of two recombinant adeno-associated viruses (rAAVs) encoding for Cas9 and gRNA. This resulted in levels of DNA editing in the tibialis anterior (13%), heart (21%) and diaphragm (5%) that were sufficient to upregulate UTRN expression by about 1.5-2-fold depending on the organs. Interestingly, this level of upregulation resulted in an amelioration of the muscle histopathological phenotype (centronucleation, necrosis, fibrosis). Additionally, we

are currently performing intramuscular injection to assess the effect of high and homogeneous editing levels in the tibilias anterior. Other gRNAs to increase UTRN expression -transcriptionally or post-transcriptionally- have also been designed and validated in human DMD myoblasts and are currently tested in mice. Overall, these findings provide the foundations for a universal gene editing therapeutic strategy for DMD.

OR079

Engineering albumin-binding domains into the capsid protein of AAV: A transient cloaking mechanism to block antibody binding for improved vector safety and efficacy

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Antibodies to recombinant AAV vectors block target cell transduction and potentiate immune effector functions such as complement activation implicated in immunotoxicity in human gene therapy. In a vector design-based approach to block antibody binding, the AAV capsid was modified by insertion of albumin binding domains (ABDs) into the common VP region. Non-covalent binding of albumin to sites distributed over the AAV surface is hypothesized to block antibody binding but not target cell transduction (Wright, 2023). Feasibility of the approach is supported by *1)* the occurrence of naturally evolved ABDs in bacteria thought to increase virulence by evasion of host antibody binding – a microbial *'wolf in sheep's clothing'*; and *2)* the successful engineering of bacterial ABDs into the repeating hexon protein of type 5 adenovirus (Rojas *et al.*, 2016) resulting in increased resistance to neutralizing antibodies. 16 AAV capsids variants ('AAVCap$_{GL}$') composed of 4 unique ABDs ranging in length from 26 to 54 aa and binding affinities from K_A ~10^8 to ~10^{10} M^{-1} were genetically inserted into 4 sites corresponding to AAV surface loops. AlphaFold software was used to generate *in silico* AAV vector structures for wild type and these 16, novel variants (AAV Cap$_{GL001\text{ - }016}$), providing a ranking of their folding viability. AAV vectors were generated by transfection of HEK293 cells using packaging plasmids (pRepCap$_{GL001\text{-}016}$) encoding ABD-modified capsid and purified by CsCl ultracentrifugation. Inefficient packaging of the AAVCap$_{GL}$ variants in a first-round production with the pRepCap$_{GL}$ plasmids alone prompted admixing of a fraction (25%) of pRepCap$_{WT}$ to facilitate capsid assembly. In the second round, one batch using pRepCap$_{GL001}$ corresponding to a 46 amino acid ABD from streptococcus protein G inserted at G453 (VP1 numbering) resulted in strong vector and empty capsid bands after CsCl. Analysis by SDS-PAGE revealed the presence, in addition to VP1, VP2, and VP3 proteins with canonical MWs, higher MW protein bands consistent with the ABD incorporation. The putative 'VP3+ABD' band was characterized by mass spectroscopy, confirming presence of sequences corresponding to *1)* VP sequences amino (N) and carboxy (C) -terminal to the ABD insertion site; *2)* chimeric VP-ABD and ABD-VP sequences at the N and C -terminal ends of the insertion site; and *3)* an internal ABD sequence. The novel vector, AAVCap$_{GL001}$, contained ~20% of ABD-modified VPs based on gel band staining, and transduced HeLa cells. This study provides proof of concept for insertion of ABDs into the AAV2 common VP region. Further studies are in progress to *1)* increase the stoichiometry of ABD incorporation into AAVCap$_{GL001}$ VP proteins; *2)* characterize albumin binding parameters; and 3) assess resistance to antibody neutralization in the presence of albumin. Further optimization of albumin-binding AAV variants has potential to improve safety, efficacy, and enable serial re-administration for AAV-based human gene therapy.

OR080

Co-stimulatory blockade regimen prevents anti-transgene and anti-vector immune responses in hemophilia A mice after *in vivo* LV gene therapy

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Lentiviral vector (LV)-based in vivo gene therapy is attractive for its potential applicability to permanently correct monogenic diseases, even in childhood, with negligible limitations derived from pre-existing immunity towards the parental virus. Although stable transgene expression by hepatocytes have been achieved in mice, dogs and non-human primates by a single systemic LV administration, immunity towards transgene and vector may limit the therapeutic outcome and LV redosing.

In a mouse model of Hemophilia A (HA), LV-mediated factor VIII (FVIII) expression by hepatocytes revealed that multiple factors may contribute to its immunogenicity, such as the protein structure, the proportion of transduced hepatocytes, the translation rate and the protein half-life. High dose of LV encoding for human (hu)FVIII (~5X10^{10} TU/kg) in adult HA mice established stable expression of huFVIII (50% of normal) in absence of anti-FVIII immune responses in 80% of treated mice, by a combination of fasting and type-I interferon (IFN) signaling blockade (anti-IFNa receptor, aIFNAR) which allowed maximizing LV transduction efficiency and dampening innate immunity. Conversely, administration of LV alone, did not result in detectable huFVIII and induced anti-FVIII immunity. Comparable doses of LV encoding for half-life extended huFVIII.XTEN resulted in minimal and only transient FVIII expression in vivo, again abrogated by anti-FVIII immunity. In order to improve FVIII output in vivo at lower LV doses, the transgene sequence was codon-optimized (coFVIII) enabling 10-fold increased expression compared to huFVIII. This strategy allowed to obtain high levels of circulating FVIII produced by a limited proportion of hepatocytes, but resulted in the induction of a strong anti-FVIII humoral and cellular immune response leading to clearance of LV-corrected hepatocytes. Therefore, we tested T cell targeted immunotherapies to control anti-transgene immunity. We found that the administration of anti-IFNAR immediately before coFVIII-LV or coFVIII-XTEN-LV administration combined with co-stimulation blockade regimen allowed stable huFVIII expression at 110% and 500% of normal, respectively, in HA adult mice by lower LV doses (~2X10^{10} TU/kg). This immunotherapeutic regimen abrogated the induction of anti-FVIII antibodies and CD8+ T cell allowing persistence of LV-modified hepatocytes releasing FVIII in absence of antibody-mediated neutralization, largely beyond the time of its administration. Furthermore, we did not detect any neutralizing anti-VSV.G humoral response, which is typically induced by in vivo administration of VSV.G-pseudotyped LV. Overall, these data indicate that FVIII immunogenicity can be controlled by i) increasing the proportion of transduced hepatocytes and lowering the amount of huFVIII produced *per* cell and ii) applying a co-stimulation blockade regimen to achieve stable FVIII expression at therapeutic levels even increasing the rate of transgene translation in a reduced fraction of hepatocytes. Moreover, the use of the latter strategy allows to adjust transgene output by administering a second LV infusion thanks to the absence of immunity to FVIII or LV particles.

OR081

Assessing molecular mechanisms of microglial mediated inflammation in retinal gene therapy

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Microglia play a major role in orchestrating the inflammatory response in neurodegenerative diseases. Thus, immunomodulatory gene therapy strategies targeting microglia hold great promise. Nevertheless, microglia are capable of mounting an immune response against adeno-associated viruses (AAVs), thus compromising their safety and efficacy profile. To identify suitable AAV serotypes for microglial gene therapy development, we transduced human induced pluripotent stem cell (hiPSC) derived microglial cells with AAV2, AAV6 and AAV8 respectively. AAV6 induced the strongest polarisation of microglial cells towards an inflammatory state as determined by morphometric analysis. We could also confirm the dose dependent nature of AAV6 mediated microglial activation. To test the relevance of our *in vitro* results in a large animal model, we performed sub-retinal injection of AAV6 and AAV8 in Yucatan minipigs and assessed its effects on retinal immune cell activation. We observed an inflammatory response in the minipigs injected with AAV6-CAG-eGFP, while minipigs injected with AAV8 and physiological saline did not exhibit adverse effects. Furthermore, in agreement with the *in vitro* hiPSC derived microglial cell model, IBA1+ mononuclear phagocytic cells in the AAV6 injected pigs retinas transitioned into an inflammatory state. The presence of the AAV genome and GFP transcripts could be detected by RNAscope in these inflammatory IBA1+ cells. Nevertheless, none of the IBA1+ cells, containing GFP transcripts, were fluorescent under confocal microscopy, thus suggesting a block in successful AAV transduction of microglial cells at the post-transcriptional level. These results highlight the importance of employing relevant *in vitro* and *in vivo* models to study AAV mediated retinal inflammation and the need to comprehend the mechanistic basis of AAV serotype specific immune responses.

OR082

Immunological Safety of Adenoviral Vector Gene Therapy

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Immunological safety is a one of the major concerns with the systemic intravascular administration of viral vectors in gene therapy. Targeting specific organs and cell types while surpassing uptake by the liver requires infusion of substantial doses of viral vectors. Introducing large quantities of viral vectors into the bloodstream poses the risk of eliciting severe and potentially fatal cytokine responses in some patients. The unpredictability and intensity of

systemic inflammatory response, coupled with a limited understanding of the molecular mechanisms behind the innate immune and cytokine responses to viral vectors, present significant obstacles to the development of new treatments, pushing the field to consider non-viral delivery methods. Nevertheless, we are confident that viral vectors, particularly adenovirus-based vectors, can provide a targeted, safe, and efficient approach to gene therapy. We hypothesize that pre-existing neutralizing and non-neutralizing antibodies, together with the complement system, influence the activation of phagocytic myeloid cells, potentially inducing a hyperactivation state and the release of pro-inflammatory cytokines. To test this hypothesis, we immunized groups of mice with human adenovirus serotypes from species B (HAdv-B3, HAdv-B11), C (HAdv-C2, HAdv-C6), and D (HAdv-D28), followed by intravenous administration of HAdv-C5-based adenovirus vectors. Whereas the antibodies generated in these mice did not have any direct neutralizing activity to HAdv-C5, the amounts of inflammatory cytokines released into the blood 6 hours post HAdv-C5 vector administration varied dramatically between mouse groups pre-immunized with different virus serotypes. Further, mice immunized with adenoviruses phylogenetically distant from HAdv-C5 produced antibodies that could bind to HAdv-C5 viral particles and trigger C3 complement fixation. We termed these antibodies cryptic non-neutralizing opsonizing antibodies (CON-Abs). Importantly, CON-Abs-mediated C3 opsonization of HAdv-C5 viral particles potentiated inflammatory cytokine release from human PBMCs in vitro. While immunization with HAdv-C5 led to the generation of virus-specific neutralizing immunity in both wild-type and C3-KO mice, the intensity of the cytokine storm following HAdv-C5 vector administration was markedly greater in C3-KO mice, indicating that complement C3 in vivo plays a role in suppressing the inflammatory response. We found that in the absence of C3 the virus is accumulating in larger quantities in CD169 positive macrophages, suggesting that C3 opsonization targets adenovirus-immune complexes away from immune phagocytic cells to non-responding cell compartments. Our findings underscore the unanticipated role of CON-Abs in moderating the severity of cytokine storm responses and highlight the need for novel approaches to pre-screen patients for pre-existing humoral factors that could amplify inflammatory reactions to therapeutic vectors.

OR083

T cell therapy for PDAC: overcoming resistance by tumor/stroma dual-targeting CAR-T cells

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PDAC is among the most lethal tumors, mostly diagnosed at advanced metastatic stages with a median survival of 8-11 months with current treatments and a 5-year survival rate of 10%. It is mostly resistant to chemotherapy and radiotherapy because of a prominent desmoplastic and

immunosuppressive microenvironment. Among promising alternatives, adoptive T cell therapy provides the possibility to replenish patient's immune system with tumor-specific cytotoxic effectors able of therapeutic effects, and of patrolling against recurrence. Attempts against PDAC have shown promising results in preclinical settings, and partial efficacy in ongoing clinical trials, sometime with associated toxicity. Choosing strategies suitable to overcome immunosuppressive and desmoplastic milieus and most appropriate target antigens represent important challenges. To advance the cure of metastatic disease, we developed a novel preclinical mouse model enabling non-invasive quantitative imaging of metastasis advancements and sensitivity to therapy. We adopted intra-portal seeding of murine PDAC cells expressing the human tumor-associated Carcinoembryonic Antigen (CEA) and traced metastasis progression by longitudinal Magnetic Resonance Imaging. PDAC lesions progressively developed over time closely mimicking the human pathology and eventually hindering liver function and mice survival. We then subjected mice to CEA-specific second-generation Chimeric Antigen Receptor (CAR) T cell therapy and found that anti-hCEA CAR T cells efficiently rejected 10days-old micrometastatic lesions and cured up to 50% of mice with 15days-old ones. In contrast, anti-hCEA CAR T cells proved ineffective against more established 21days-old lesions. Of note, up to 50% of day 15-treated animals showed evidence of unchecked disease progression, suggesting that by this time mechanisms of suppression emerged. Mechanistically, we found regulatory T cells to be highly enriched within liver metastases and in the surrounding area compared to adjacent normal tissue. Concomitantly we found integrin avb6 expression and active TGFβ-signaling to define growing metastatic lesions. Given that TGFβ and T regs are major drivers of desmoplastic reactions and of immunosuppressive microenvironment in PDAC, that activation of latent TGFβ is primarily mediated by the avb6 and avb8 integrins, also negative prognostic indicators for PDAC, and that avb8 is expressed by T-regulatory cells, we reasoned these integrins might represent ideal novel CAR-T cell targets. To simultaneously target the two integrins we took advantage of a chromogranin A-derived peptide with dual avb6/avb8 specificity to design novel peptide-driven mouse and human CAR constructs and engineer T cells (pepti-CAR T). We validated specificity on recombinant integrins and tumor cells *in vitro*, and efficacy against subcutaneous PDAC lesions *in vivo* in immunocompetent and immunodeficient settings. Towards clinical implementation we investigated efficacy against Patients Derived PDAC Organoids in NSG mice and found pepti-CAR able to reject orthotopic lesions. When tested in immune reconstituted NSG mice, pepti-CAR-T cells lowered the frequency of intratumoral $CD4^+CD25^+FOXP3^+$ T regulatory cells, suggesting their specific targeting. Thus, our data identify windows of sensitivity/resistance to CAR-T cell therapy within liver PDAC metastases, and their overcoming by CAR-T cells endowed with tumor/stroma dual-targeting activity.

OR084

CAR-Treg cell therapy to induce tolerance in liver transplantation – LIBERATE clinical trial

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Regulatory T cells (Tregs) are essential for the maintenance of immune tolerance and to regulate inflammatory responses. Multiple pre-clinical models of transplantation have demonstrated that Tregs can mediate donor-specific responses and induce allograft acceptance. Consequently, the clinical use of Treg cells targeting donor antigens provides an attractive therapeutic approach to induce transplantation tolerance and to eliminate the burden of life-long pharmacological immunosuppression.

LIBERATE is a single-arm, open-label, multi-center, first-in-human, phase I/II clinical trial that is investigating the use of autologous anti-HLA-A2 CAR-Tregs (QEL-001) in HLA-A2 negative adult liver transplant patients that received a graft from an HLA-A2 positive donor (NCT05234190). A proprietary GMP manufacturing process has been developed to engineer recipient Treg to express the anti-HLA-A2 CAR, a FOXP3 phenotype lock and a safety switch. QEL-001 CAR-Treg show consistent expression of all 3 transgenes, while preserving the transcriptional and protein expression patterns of non-transduced Tregs. Hallmark Tregs-associated markers such as FOXP3, HELIOS and CTLA4, together with persistent demethylation status of the TSDR region of FOXP3 gene and minimal expression of pro-inflammatory cytokines characterise the phenotype of the QEL-001 product.

The clinical batches from the LIBERATE safety cohort showed successful engineering and expansion of liver transplant Tregs, generating drug products according to the release specifications. QEL-001 was well tolerated by all patients in the safety cohort, and no adverse events (AE) related to cell products were reported. Extensive immunomonitoring analysis has been performed from blood samples and liver tissue biopsies to assess product safety and potential therapeutic efficacy. Characterization of CAR-Tregs in circulation has shown cell persistence post infusion, maintaining the expression of proteins associated to their suppressive phenotype such as FOXP3 and HELIOS. In addition, analysis of liver biopsies 28 days post infusion confirmed trafficking, engraftment, and phenotypic stability of the QEL-001 product in the transplanted livers.

Overall, we have demonstrated that Tregs from liver transplant patients can be isolated and expanded to generate engineered CAR-Tregs with enhanced safety and functional characteristics. The products infused to patients in the LIBERATE safety cohort have shown to be safe and well tolerated. CAR-Tregs were able to engraft and persist while maintaining their suppressive phenotype, providing key evidence to support further evaluation of the use of CAR-Tregs to induce operation tolerance in liver transplant patients.

OR085

Novel CD33/CLL-1-directed dual CAR-T cells mediate potent antigen-specific cytolytic activity in mouse models of Acute Myeloid Leukemia (AML)

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AML is the most common form of acute leukemia in adults. However, current therapies rarely yield durable remissions, with >40% of patients relapsing within 3 years. The poor clinical outcome for AML patients reinforces the urgent need for the development of more durable therapeutic approaches. Two AML-related antigens have been targeted separately in CAR-T cell clinical trials, since they are both highly expressed on AML blasts and leukemic stem cells: CD33 (e.g.: NCT05984199, NCT04849910) and CLL-1 (CD371, NCT04789408). Single-antigen targeting, however, can cause antigen-negative variant selection and antigen escape leading to relapse, especially given the heterogeneity of AML within and across patients. Here, we present preclinical data detailing the development and functional characterization of novel multi-specific CAR-T cells directed against CD33 and CLL-1 with a logical “OR” gate to protect against possible antigen escape mutants.

To identify the most optimal multi-specific CAR format, we initially compared CAR-T cells expressing tandem (two binders in one CAR construct) and dual (two independent CARs, each with one binder) constructs with validated binders against CD33 and CLL-1. Each construct was transduced into primary T cells using lentiviral vectors and investigated for antigen-specific AML cell killing, for potency at low effector to target cell ratios, and for long-term persistence in repeated stimulation assays. Dual format CAR-T cells displayed 1.5-fold greater potency and 3-fold higher persistence *in vitro*. All multi-specific CAR formats were further validated in an *in vivo* murine xenograft model of AML. Importantly, tandem CAR-T cell infused mice presented AML progression, while dual CART-cell treated mice displayed complete and sustained clearance of AML, supporting their superior anti-tumor activity. We then generated 18 dual CAR constructs with three different co-stimulatory domain permutations, relying on two CD33 and three CLL-1 binders, which were chosen in separate single-target CAR-T screens of 18 and 24 candidates, respectively. All constructs were assessed for T cell expansion and CAR expression in three distinct primary T cell donors. Only the 12 constructs that exhibited potent target cell lysis and CAR-T cell cytokine secretion in the presence of both or either antigen, with minimal nonspecific killing, were further tested in multiple promyeloblast HL-60-based *in vivo* models of AML. The final four lead dual candidates significantly reduced tumor growth, induced T cell expansion, extended animal survival, presented greater persistence in hematological compartments, and showed osteotropic activity.

Altogether, our preclinical data demonstrate highly efficacious dual CAR-T cells against CD33 and/or CLL-1, with potent *in vitro* and *in vivo* cytolytic activity. The presented multi-specific CAR-T cell approach targeting two different AML antigens can cover a larger patient population and induce a more robust and durable anti-leukemic response by addressing AML antigen heterogeneity and antigen escape. These results support further clinical development of the lead dual CAR candidates, either as a stand-alone treatment or in combination with Vor's platform of engineered transplant to eliminate on-target, off-tumor toxicity of CAR-T cells to fully benefit high-risk AML patients.

OR086

Molecular mechanisms promoting long-term cytopenia after BCMA CAR-T therapy in Multiple Myeloma

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Hematologic toxicity, specifically prolonged cytopenia, is a common side effect associated with CAR-T therapies, being particularly severe in patients with relapsed/refractory Multiple Myeloma

(MM). However, to date, the mechanisms responsible for this complication remain largely unknown. Thus, the aim of this study was to investigate and understand the underlying mechanisms of prolonged cytopenias associated with CAR-T therapy targeting BCMA.

In our work, we combined the retrospective analysis of a cohort of 48 patients treated at Clinica Universidad de Navarra with BCMA CAR-T cells, with *ex vivo* transcriptomic analysis using single-cell RNA sequencing, to characterize the kinetics of cytopenia, identify predictive factors and determine potential mechanism underlying these toxicities. The overall incidence of cytopenia was 95.7%, and grade>3 thrombocytopenia and neutropenia, one month after infusion, was observed in 57% and 53% of the patients, being still present after one year in 4 and 3 patients respectively. Presence of cytopenia at baseline and high peak inflammatory markers highly correlated with cytopenia persisting up to three months.

To determine potential mechanisms underpinning cytopenias, we evaluated the paracrine effect of BCMA CAR-T cells on HSPCs differentiation, using an *ex vivo* myeloid differentiation model. Thus, supernatants from CAR-T cells or control T lymphocytes co-cultured with U266 tumor cells were added to the differentiation process. Phenotypic analysis by flow cytometry showed that supernatants from activated CAR-T cells (spCAR) halted HSPCs differentiation, promoting more immature phenotypes, with reduced expression of granulocytic, monocytic, and erythroid maturation markers, suggesting a paracrine effect related to CAR-T cells activation. Interestingly, this phenotype could be prevented by a combination of IFNγ, TNFα/β, TGFβ, IL-6, and IL-17 inhibitors, indicating the essential role of these cytokines and providing potential targets to prevent these cytopenias. Transcriptomic analyses at single cell level (scRNA-seq), performed using 10X Genomics technology, revealed that cells differentiated in the presence of spCAR were enriched in transcription factors related to early stages of hematopoietic differentiation, such as *GATA2 RUNX1* and *CEBPA*. Additionally, gene regulatory network (GRN) analysis identified, analyzed using SimiC, identified decreased activity of key regulons involved in neutrophil and monocytic maturation, such as *MAFB* and *ID2*, providing insights into the molecular mechanisms responsible for the observed phenotypic changes.

Our results suggest that CAR-T cell activation negatively influences hematopoietic differentiation through paracrine effects inducing HSPCs maturation arrest. Moreover, our study contributes to the understanding of mechanisms promoting severe cytopenia observed after CAR-T therapy in MM and provides potential treatments to prevent or decrease its severity.

OR087

Elimination of cellular HIV reservoirs by CCR5/CD45 multiplex base edited CD45 CAR-T cell therapy

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Eradicating cellular reservoirs of human immunodeficiency virus (HIV) in patients on antiretroviral therapy (ART) is critical to curing HIV. There are rare but compelling reports of HIV cures in patients who underwent allogeneic hematopoietic stem cell transplantation (alloHSCT) from CCR5$\Delta^{32/}\Delta^{32}$ donors, wherein donor alloreactive T cells completely eradicated the recipient's immunohematopoietic system and allowed it to be replaced by donor-derived hematopoiesis lacking the vital HIV co-receptor CCR5. While applying this strategy systematically as a treatment option is not feasible due to the paucity of HLA-matched CCR5$\Delta^{32/}\Delta^{32}$donors for HIV-infected patients and the high-risk nature of alloHSCT, these reports highlight that immunologic depletion

of HIV reservoirs (via alloreactivity) in combination with an HIV-resistant HSCT (via CCR5 deficiency) can achieve long-lasting HIV remission. Here, we sought out to mimic these "experiments of nature" in an autologous setting, thereby increasing the safety and accessibility of this one-time functional cure for HIV by developing an HIV- and fratricide-resistant CAR-T cell therapy against the pan-leukocyte antigen CD45 that can eliminate the cellular HIV reservoir which is paired with an HIV- and CART45-resistant HSCT to preserve the hematopoietic system.

Fratricide-resistance of the anti-CD45 CAR-T cells is achieved by base editing the targeted epitope on CD45 to prevent CAR recognition while maintaining CD45 expression and function. We hypothesized that HIV resistance can also be achieved via base editing by removing the start codon of CCR5, thereby preventing its translation, and mimicking the natural loss of CCR5 expression in individuals with the CCR5$\Delta^{32/}\Delta^{32}$ mutation, or by introducing point mutations at sulfotyrosine residues (Y14 and Y15) in CCR5's N-terminal domain that are critical for binding to HIV-gp120. Using a PAM-relaxed NG-ABE8e editor with a CCR5 start codon targeting sgRNA, we achieved editing efficiencies of up to 84% in primary human T cells. Similarly, base editing to install Y14H and/or Y15H mutations was also highly effective in primary human T cells with editing efficiencies of ~90% for A5 (Y14H) and ~38% for A2 (Y15H).

We demonstrate that either CCR5 modification achieves HIV resistance and in vitro and in humanized mouse models, with N-terminal mutagenesis of CCR5 accomplishing transient resistance while preserving CCR5 expression. Utilizing scDNA sequencing, we further show that CCR5 base editing can be multiplexed with CD45 epitope editing with a co-occurence of biallelic editing at the targeted CCR5 and CD45 loci in ~90% of the cells.

Next, we combined CD45/CCR5 base editing with lentiviral transduction of a previously developed CD45-directed CAR to generate HIV- and fratricide-resistant CAR-T cells and show that they effectively eliminate HIV-infected CD4 T cells in xenograft models while protecting the engrafted CAR-T cells from infection. In contrast, infection readily spread to CCR5 WT CART45 cells, suggesting that multiplex editing is required to maintain adequate T cell numbers.

Overall, our study demonstrates the potential of anti-CD45 immunotherapy to eradicate HIV infected cells. We hypothesize that when combined with a CD45/CCR5 edited autoHSCT, this approach can regenerate an HIV- and anti-CD45 resistant hematopoietic system free of latent HIV infected cells, thus laying the groundwork for a one-time functional cure.

OR088

Successful Preclinical Proof-of-Concept Study of a CAR-T Cell Approach Targeting CD84 to Treat Acute Myeloid Leukemia

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Chimeric antigen receptor (CAR) T cell therapy has proven highly effective in treating relapsed/refractory B cell malignancies, including pediatric acute lymphoblastic leukemia. Efforts to replicate this success in other hematologic and solid tumors are actively underway. Main

obstacles for CAR T cell therapy in T cell malignancies and acute myeloid leukemia (AML) include antigen specificity, off-tumor toxicity, and persistence.

With the goal of developing a novel and highly specific CAR-T cell approach for AML, we performed a large analysis of a gene expression dataset from AML patients and identified for the first time the CD84 as an AML specific antigen. CD84 is highly expressed on AML blasts of 99% of the tested validation cohort composed of newly diagnosed and relapsed pediatric AML patients. This antigen is also characterized by a very low/null expression on healthy regenerating bone marrow cells and hematopoietic stem cells. Two novel single-chain variable fragment (ScFv) recognizing CD84 were thus generated by phage display. The new CAR cassettes containing these ScFv chains were cloned into a plasmid transfer to produce third-generation lentiviral vectors (LVs). We then applied a standard protocol for producing second-generation 4-1BB CAR-T cells by LV transduction of IL-7 and IL-15 activated T cells. After 14 days of expansion, the CAR-T cells generated at a >20 fold expansion rate were mostly $CD4^+$ (>70%), with central memory (Tcm, 65% of $CD4^+$ cells), *naïve* (Tn) and stem cell memory (Tscm) differentiation (cumulatively >20% of $CD4^+$ cells), and no effect of the ScFv chains on cell activation or exhaustion. The CAR-T cells were co-cultured with several target cell lines expressing CD84 and non-target $CD84^{neg}$ cell lines, and on *ex vivo* cells from pediatric patient-derived xenograft (PDX) models to assess specificity, at 1:1 effector to target (E:T) ratio. After 48 hours of co-culture, CAR-T lytic activity was analyzed by flow-cytometry with Annexin V and 7-AAD, and by luciferase viability assay on luciferase-transduced AML cell lines. We documented a significant i*n vitro* antitumor activity with a lysis potency >50% on target AML cell lines and primary AML. Importantly, the CAR-T cells did not exert toxicity on hematopoietic stem and progenitor cells, as they induced neither a significant reduction in the number of colony-forming units (CFUs) generated by $CD34^+$ cells nor a reduction in cell viability, both *in vitro* and in humanized $CD34^+$ NSG models. To evaluate the anti-leukemia activity of anti-CD84 CAR-T cells we employed three AML-PDXs characterized by different CD84 expression levels (3:1 E:T ratio). In mice treated with the empty CAR-T cells bioluminescence appeared as early as week 4 after inoculation and increased rapidly, whereas treated mice showed a complete and significant persistent reduction of leukemia burden to undetectable levels (by monitoring both luciferase and $CD45^+$ cell counts weekly in peripheral blood). Yet, anti-CD84 CAR-T cells induced a significantly increase in survival of PDX mice, attaining >40 weeks of observation in the model with highest CD84 expression ($p<0.0001$).

Overall, this proof-of-concept study provides support to develop such immunotherapy approach based on targeting CD84 to treat AML.

OR089

Peptide-assisted tethering of DNA repair effectors to Cas9 for precise genome editing

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CRISPR-Cas9 has emerged as a versatile gene editing tool in the field of cell and gene therapy. Its ability to induce dsDNA breaks can be exploited to revert a disease-causing mutation via the homology-directed repair (HDR) pathway. However, such strategies suffer from the intrinsically low frequency of HDR compared to the alternative non-homologous end-joining (NHEJ) repair pathway at target site. Efforts to enhance HDR efficiency include the use of drugs, that inhibit NHEJ, or the development of Cas9 fusion proteins, that alter the normal resolution of the Cas9-

induced DNA break to eventually favour HDR. While promising, the efficacy of these approaches varies greatly across cell types, possibly related to different expression profiles of DNA repair factors.

To address cell type specificity, we introduce an innovative CRISPR Cas9 strategy designed to exploit the dimerization capability of synthetic peptides. The proposed CRISPR Peptide Assisted Localization (PAL) system comprises two essential elements: 1) a Cas9 nuclease fused to a small synthetic peptide and 2) a library of DNA repair effectors fused to a complementary peptide.

Here, we demonstrate how CRISPR-PAL can be used to identify the most effective combination of effectors tailored to a particular cell type. Distinct nuclease and effector combinations resulted in up to a 3-fold increase in HDR events compared to unmodified Cas9 across diverse cell types. Furthermore, the dimerization capability of CRISPR-PAL system enabled the use of novel combinations that were unamenable through mere overexpression of effectors. Our findings highlight the versatility of this system which can be optimized to maximize precision editing across a wide range of cell types. Ongoing experiments explore the efficacy of the CRISPR-PAL system in correcting Fanconi Anemia (FA) specific mutations by enriching the missing effectors at the target site in patient-derived cells. We anticipate that the CRISPR-PAL approach will prove instrumental in the development of more effective, tailored genome editing solutions with broad therapeutic applications.

OR090

Genome Editing of Human APOE4 to APOE3 in the Brain of APOE4 Mice

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Apolipoprotein E (APOE) is a 299 amino acid lipid transport protein that is the major carrier of cholesterol in the CNS. Common genetic variants of APOE are major risk factors for the development of Alzheimer's disease (AD), with APOE3 associated with an average risk, APOE2 a reduced risk and APOE4 an 8 to 12-fold increased risk, with APOE4 homozygotes developing earlier onset and more severe AD. There is extensive biochemical, molecular and physiologic data supporting the concept that APOE4 is toxic to the CNS. In this context, and with the knowledge that APOE4 (Arg112) and APOE3 (Cys112) differ by only a single amino acid, we hypothesized that we could use prime editing to genetically convert APOE4 (Arg112 CGC) in the brain to APOE3 (Cys112 TGC), therefore theoretically reducing the risk of APOE4 homozygotes for the development of AD. A series of epegRNAs, reverse transcriptase templates and nickcase candidates were screened to determine the optimal combination for efficient cas9-mediated prime editing. The initial studies were carried out in the human APOE4 homozygote U937 monocytic cell line. The efficiency of editing was initially determined by allele-specific PCR followed by confirmation by high throughput sequencing. The efficiency of conversion of APOE4 to APOE3 [% editing = E3/(E4+E3)] ranged from 2.4% to 77.8% depending on the editing construct. Following selection of the most efficient candidate editing constructs, 2 serotype rh.10 adeno-associated vectors were used to deliver prime editor components using NpuN split-intein system [AAVrh.10 construct 1 (N-term of Spcas9); AAVrh.10 construct 2 (C-term of Spcas9_PE2max)]. Two selected editing candidates were delivered intravenously (n=5) or to the hippocampus (n=5) of APOE4 humanized TRE4 mice (human APOE4 replacing mouse ApoE under control of the mouse ApoE promoter), all mice receiving a total of $2x10^{12}$ vector genomes. In the intravenous administered group, there was efficient editing in the liver with the two selected

candidates having 30.0%±2.7 and 16.9%±1.4. Strikingly, in the brain at the site of treatment in the hippocampus, editing efficiency for the same candidates were 17.7%±1.8 and 7.3%±1.0 respectively. These observations support the feasibility of developing *in vivo* brain gene editing to convert APOE4 to APOE3, helping to protect the CNS of APOE4 homozygotes from the development of Alzheimer's disease.

OR091

Mutation-independent genome editing approaches for treatment of Stargardt disease

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Genome editing via the CRISPR-Cas9 system is an exciting field of biomedical research which is gaining increasing interest for treatment of several diseases. However, classical genome editing approaches still have limitations, including: i. sequence-specificity, that limits its applicability to diseases with high allelic heterogeneity; ii. inability of the currently available techniques to achieve efficient precise integration of a corrective DNA template in post-mitotic cells. These limitations have thus far hindered development of effective gene editing approaches for several inherited retinal diseases, including the most common form of inherited macular degeneration, Stargardt disease (STGD1), which is due to more than 1200 different mutations in the large retinal-specific *ABCA4* gene. Therefore, in this project, we aim to develop novel adeno-associated viral (AAV) vector-based genome editing strategies for integration of large templates in *ABCA4*, to allow correction of multiple mutations with a single therapeutic DNA template. To achieve integration of the template we planned to explore different repair pathways which are active in post-mitotic cells like photoreceptors, the target cells for STGD1 treatment. Accordingly, we have developed multiple candidate sets of AAV-Abca4 vectors for targeting either the murine or the human *ABCA4* gene. We side-by-side compared the different approaches *in vitro*, in both murine and human cell lines, and found that they effectively mediate template integration at the endogenous locus, resulting in *ABCA4* expression. Subretinal delivery of the AAV-based genome editing approaches in the retina of *Abca4-/-* mice results in targeted DNA template integration and reconstitution of *Abca4* expression in transduced photoreceptors at levels that were found to ameliorate the STGD1 phenotype in *Abca4-/-* mice. Further evaluation of the efficacy of our strategies in human iPSC-derived 3D retinal organoids from STGD1 patients is ongoing.

OR092

Novel inverted terminal repeat sequences and flanking proximal regions from serotypes AAV8 and AAV.rh39 show robust promoter-like activities that enhance transgene expression in a tissue-dependent manner

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Strategies to boost the potencies of adeno-associated virus (AAV)-based gene therapies have mainly focused on three main facets: dose and route of delivery, capsid engineering, and promoter design. Unfortunately, advancements in these areas have reached a plateau for many desired target organs, while therapeutic efficacies still fall short for many applications. Previous research has demonstrated that inverted terminal repeats (ITRs), the last remaining viral elements in AAV vectors, harbour promoter-like activities. However, this feature has yet to be leveraged for designing better gene therapy vectors. We have now identified two new ITR sequences cloned from the full viral genomes of serotypes AAV8 and AAV.rh39 (ITR8 and ITR.rh39, respectively), and have been characterizing their functions during vector production and their impacts on transduction in mice. Within the context of packaging with AAV8 capsids, vector production was unaffected by the inclusion of these novel ITRs. These results suggest that they are fully compatible with standard triple-plasmid transfection production schemes, with no loss in titres when using Rep from AAV2 during packaging. Interestingly, ITR8 and ITR.rh39 respectively demonstrated 5-fold and 10-fold increases in vector transduction when compared against constructs harbouring the “standard” ITRs from AAV2 (ITR2). Furthermore, we have identified ~120-nt sequences present at the 3’ ends of the AAV8 and AAV.rh39 genomes called ITR-proximal regions (IPRs) that show tissue-dependent, enhancer-like activities. The full IPR8 and IPR.rh39 sequences boosts transduction in skeletal muscle following intramuscular injections, but do not significantly alter transduction in liver after intravenous delivery. Preliminary data suggests that the increases in transduction conferred by ITR8 and ITR.rh39, when compared to ITR2, are only in part due to enhanced transcription.

Our work demonstrates that engineering efforts to improve vector transduction for gene therapy can be expanded to include ITR/IPR sequences. Furthermore, these results indicate that the natural AAV virus may utilize serotype-specific mechanisms that are beyond what is defined by the capsid to establish its tropism profile and tissue reservoir.

OR093

Generation of hepatocyte organoids from primary hepatocytes

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The liver is a unique organ that fulfils many vital functions for the organism. The two main cell types of the liver are the hepatocytes (which perform most of the hepatic tasks) and the cholangiocytes (which form the bile ducts of the biliary tree). Due to their functional importance, diseases targeting the liver carry life-threatening consequences. Accordingly, liver disorders account for more than 2 M deaths annually in the world. The only treatment available for end-stage liver disease is organ transplantation, which has many associated drawbacks. Cell therapy could provide an advantageous alternative by transplanting hepatic cells that could restore the functionality of the damaged organ. Primary hepatocytes have already been used for cell therapy applications. However, they cannot be cultured extensively in vitro without losing their functionality and morphology, limiting the number of patients that can benefit from this approach. Thus, developing new conditions for the culture of primary hepatocytes in vitro has become a major research topic. The generation of liver organoids has been explored as a potential option, as they acquire higher complexity and greater maturity thanks to the cell-cell and cell-matrix interactions. Several groups have successfully developed cholangiocyte organoids, which have been employed in proof-of-principle studies for cell therapy. However, generating hepatocyte organoids remains challenging due to a lack of reproducible culture conditions and donor differences. To overcome this drawback, we have performed a screening to identify factors and basal media to maintain hepatocyte organoids in vitro. For that, we used human primary hepatocytes derived from three different donors and identified defined conditions that allow the proliferation of mature hepatocyte organoids. These organoids can be expanded for a prolonged amount of time, while maintaining the expression of mature hepatocyte markers, such as ALB. The development of reproducible methods to derive hepatocyte organoids from human primary cells can have important implications not only for cell therapy but also for drug screening and disease modelling.

OR094

Investigating the effects of progranulin reconstitution driven by microglia-directed gene therapy in iPSC-derived neural networks

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Ex vivo autologous haematopoietic stem and progenitor cell (HSPC) gene therapy, a curative approach developed for several immune and blood disorders, may also be used for partial replacement of microglia in the central nervous system with genetically modified cells. We set out to develop HSPC gene therapy for two fatal neurodegenerative diseases caused by progranulin (PGRN) deficiency, namely frontotemporal dementia and neuronal ceroid lipofuscinosis 11. Mutations in the granulin (*GRN*) gene lead to dysfunctional and neurotoxic microglia in both diseases for which no treatment is available to prevent or slow neurodegeneration. We designed self-inactivating lentiviral vectors (LVVs) carrying novel myelospecific promoters that showed potent transgene expression in macrophages derived from human myeloid cell lines and healthy human HSPCs. Our lead gene therapy LVV was next tested in patient induced pluripotent stem cell (iPSC) derived microglia, confirming significantly increased PGRN secretion. We analysed the dynamics of transgene expression during phagocyte differentiation in this model, showing that reporter expression from the integrated LVV is not detected at the pluripotent state and is progressively switched on following myeloid lineage specification. To investigate the effects of PGRN reconstitution in iPSC derived microglia, we are currently studying electrophysiological properties, phagocytic activity and cytokine response to pro inflammatory stimuli for phenotypic characterisation. We combine these functional readouts with gene expression profiling to comprehensively evaluate the phenotype of these cells and the impact of gene therapy. To model the interaction of neurotoxic PGRN-deficient microglia with other brain cells, we developed a co-culture system consisting of neurons, astrocytes and microglia differentiated from human iPSCs. We showed that highly ramified microglia integrated in the multilayered network of neurons and astrocytes within 2 weeks of co culture, mimicking the migration of microglia progenitors into the developing brain during embryonic development. In this model, we are currently investigating the impact of PGRN deficient and PGRN reconstituted microglia on neurodegenerative phenotypes such as neuronal loss, lysosomal dysfunction and accumulation of pathological TAR DNA-binding protein 43 (TDP-43) aggregates. Our findings support further clinical development of the myelospecific LVVs we have developed for *GRN*-related neurodegeneration.

OR095

Investigating the interplay between DNA repair pathways and recombinant AAV integration into CRISPR-Cas-induced double-strand breaks *in vivo*

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Adeno-associated viruses (AAVs) are promising delivery vehicles for CRISPR-Cas tools due to their ability to transduce various tissues using different capsids and their safety profile. However, they face limitations such as packaging constraints, long-term Cas expression, and genome integration at CRISPR-mediated double-strand breaks (DSBs). Here, we evaluated different computational tools for determining AAV integrations based on short-read amplicon sequencing. We also investigated the impact of DSB free-end nature and DNA repair mechanisms on AAV integration frequency.

We delivered paired-SaCas9 and Cas9 nickases via AAV to the liver of a mouse model of primary hyperoxaluria type 1 for targeted disruption of the *Hao1* gene. Both strategies achieved equivalent editing efficiency and therapeutic effect. An in-depth analysis of the raw sequencing data revealed insertions aligning with AAV genomes in both groups. However, common analysis tools underestimate AAV integration events. By optimizing alignment scoring parameters, we improved the detection of AAV insertions. Using these optimized parameters, we observed significantly lower vector sequence insertion rates in DSBs induced by paired Cas9 nickases compared to Cas9 nucleases. Given that paired Cas9 nucleases mainly generate blunt-end DSBs, while paired Cas9 nickases create staggered ends, we hypothesized that differences in AAV integration frequency are linked to repair mechanisms. We observed that staggered DSBs mediated by paired Cas9 nickases, but not nuclease-mediated DSBs, were predominantly repaired through microhomology (MH)-dependent repair pathways.

To further explore the correlation between AAV integration and MH frequency, we reanalysed short-read amplicon sequencing data from various studies utilizing different CRISPR-Cas systems delivered by AAVs *in vivo*. Preliminary data suggested that staggered ends created by Cas12 commonly resulted in high MH scores and low AAV insertion frequencies, whereas the frequency of MH-dependent repair upon blunt-ended DSB formation likely depends on the genomic context. Interestingly, AAV insertions negatively correlated with MH-dependent repair frequency. Importantly, no homology was found between AAV genomes and insertion sites. All the presented data suggest that the repair mechanism plays a role in AAV integration, and its frequency could be reduced by designing Cas9 gRNAs in regions with a high predicted microhomology score. However, the influence of other biological and experimental variables on the frequency of integration should be further investigated.

OR096

Development of an epigenome editing strategy for the treatment of β-hemoglobinopathies

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β-hemoglobinopathies, including β-thalassemia and sickle cell disease, are genetic disorders caused by mutations in the β-globin gene, leading to reduced or abnormal adult hemoglobin production. These conditions are associated with significant morbidity and mortality. Amelioration of β-globin disorders occurs when postnatal expression of fetal hemoglobin (HbF) is maintained, a condition known as hereditary persistence of fetal hemoglobin. In this context, fetal γ-globin gene expression compensates for β-globin deficiency, mitigating disease severity. Based on this observation, gene editing approaches aiming at inactivating major repressors of γ-globin gene expression (e.g., *BCL11A* and *ZNF410*) have been developed, showing promising preclinical and clinical results, with the first gene editing drug product approved on the market. However, concerns about potential genotoxicity from unintended CRISPR-Cas9 activity at on- and off-target sites highlight the need for safer gene therapy approaches.

Epigenome editing is emerging as a promising alternative to conventional genome editing by providing an efficient and DNA break-free platform for durable gene silencing. We have recently shown that a single administration of lipid nanoparticles loaded with mRNAs encoding Engineered Transcriptional Repressors (ETRs) can install efficient and long-lasting gene silencing in the mouse liver, through targeted deposition of DNA methylation. Here, we exploited dCas9-based ETRs to

reawaken γ-globin expression in erythroid cells through epigenetic silencing (epi-silencing) of *ZNF410* in human hematopoietic stem and progenitor cells (HSPCs).

To rapidly identify gRNAs for *ZNF410* epi-silencing, we developed an *in situ* saturating screening approach based on lentiviral vector-mediated gRNA expression and transient ETR delivery. Using this approach, we interrogated two annotated regulatory elements of the human *ZNF410* gene with >500 gRNAs in an *ad hoc* engineered human erythroleukemia reporter cell line that expressed tdTomato from the *ZNF410* gene. These studies led to the identification of several gRNAs that induced stable (up to 20 days) and efficient (up to 85%) epi-silencing of *ZNF410* in both the reporter cell line and HUDEP-2, an immortalized human erythroid progenitor cell line. In parallel, we optimized culture conditions and editing procedures to promote efficient epi-silencing in human HSPCs from healthy donors, using the *B2M* gene as an easy-to-measure readout. Using this protocol, we reproducibly achieved >80% of *ZNF410* and *B2M* epi-silencing in bulk-treated and phenotypically defined stem cells. Importantly, epi-silencing of both genes persisted upon *in vitro* culturing or single-cell differentiation of treated HSPCs, without altering progenitors' outputs in terms of relative cell type composition and numbers. Finally, in erythroid colonies, epi-silencing of *ZNF410* was accompanied by a >2.5-fold increase in γ-globin expression, with a concomitant reduction in β-globin. Molecular analyses indicated near-complete *de novo* methylation of the *ZNF410* promoter in ETR-treated cells.

Overall, we showed here that transient ETR delivery in primary HSPCs leads to efficient silencing of *ZNF410 in vitro*, withstanding the extensive epigenetic rewiring programs of terminal hematopoietic differentiation and resulting in increased γ-globin expression. Ongoing xenotransplantation studies in mice will unravel the efficiency and durability of *ZNF410* epi-silencing in long-term repopulating HSPCs with implications for clinical translation.

OR097

Gene correction of HIGM1 CD4+ T cells: a comprehensive analysis of GMP-compliant process performance and product critical quality attributes

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CD40 Ligand (CD40L) is a costimulatory molecule mainly expressed by CD4 lymphocytes upon activation in a transient and tightly regulated manner. CD40L binds cluster of differentiation 40 (CD40), a receptor expressed by B lymphocytes and other cells of the immune system. Mutations in CD40L gene (*CD40LG)* compromise the crosstalk between activated $CD4^+$ T cells and effector cells thus impairing the development of a complete and adequate immune response against specific antigens. Loss of function mutations in *CD40LG* cause Hyper IgM Syndrome 1 (HIGM1), a rare X-linked combined immunodeficiency. Using a knock-in gene editing (GE) strategy based on CRISPR/Cas9 and integrase-defective lentiviral vector (IDLV) delivery of the corrective template, we have recently developed a scalable good manufacturing practice (GMP)-compliant process that allows for correction, selection and expansion of edited cells. With the aim of characterising the final product and to evaluate the overall performance of the manufacturing process, standardised and reproducible quality control (QC) methods were designed, considering all elements that may have an impact on the quality of the products. In view of the clinical translation, methods have been validated according to EMA/CHMP/ICH/172948/2019 ICH guideline M10 and to FDA Bioanalytical Method Validation Guidance for Industry. To verify the process suitability for the upcoming clinical trial, $CD4^+$ T cells derived from a cohort of patients aged 2-54 were corrected, in accordance to the optimised manufacturing protocol, and extensively characterised. As observed with Healthy Donors (HDs), patient cells were edited with high efficiency, maintained $CD4^+$ cell purity and clonal diversity until the end of the process and showed a high proportion of T stem cell memory (TSCM) cells. Nevertheless, patient cells displayed a slightly different proliferation kinetic with a higher initial proliferation rate, followed by a lower one. This resulted in an overall slightly lower process yield probably related to the differences between HIGM1 patients and HDs in terms of CD4+ subset distribution. As for HDs cells, analysis of activation/exhaustion markers at the end of the manufacturing process showed very few double positive cells and none triple or quadruple positive for the markers analysed, suggesting that the culture conditions did not drive cell exhaustion. No cytokine-independent growth was observed and no large deletions at target site were detected. Importantly, corrected patient cells displayed restoring of regulated *CD40LG* expression, binding to CD40, and downstream signal transduction. No process related impurities including residual Cas9 and residual IDLV donor template were present at the end of the process. Taken together these data provided valuable information for tailoring process with real-word patient cells and demonstrate the feasibility of GMP-compliant large scale manufacturing of gene corrected HIGM1 $CD4^+$ T cells at clinical-grade quality.

OR098

Scaling up manufacturing to 1000L and beyond with novel AAV capsids and payloads: From early developability assessment to process development and scale-up

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Scalability to large scale manufacturing remains a challenge in meeting AAV material demand for the clinic and poses a hurdle to successful commercialization of gene therapy products. While there has been significant progress made in advancing manufacturing of gene therapies, particularly with HEK293 based suspension processes, there is still opportunity for further improvements.

We start with early developability assessments to evaluate novel potential candidates on their likelihood to advance towards the clinic by being a manufacturable, safe and efficacious drug. Specifically, the payload concentration and sequence, capsid occupancy, integrity, sequence, aggregation state and functional output were examined using multiple techniques to provide a holistic view of the different candidates during early development. Detailed analytical characterization distinguished key methods that were able to provide early indicators of loss of structural integrity. Notably ddPCR, AUC and SEC-MALS-FLD indicated the impacts of different capsid/transgene combinations and storage conditions.

Subsequently, the lead candidate was taken through small scale process development and was successfully scaled up with multiple large scale runs. The manufacturing process uses HEK293 in suspension with transient transfection with a chromatography based downstream process. Design of Experiments (DOE) studies on various cell culture process parameters were conducted to define the process leading to consistently high titers. The downstream process uses an affinity capture step followed by a polishing chromatography step to achieve > 70% full capsids. In addition to process yield comparisons across small- and large-scale batches, detailed analytical results including AUC analysis, aggregation by SEC and in-vitro potency demonstrates scalability and commercial viability of the manufacturing process.

OR099

Atidarsagene autotemcel (lentiviral hematopoietic stem cell gene therapy) for late juvenile metachromatic leukodystrophy: preliminary results from a phase III clinical trial

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Metachromatic Leukodystrophy (MLD) is a rare, disabling and fatal lysosomal storage disease. Atidarsagene autotemcel (arsa-cel) is a hematopoietic stem cell (HSC) gene therapy (GT) and consists of autologous $CD34^+$ cells transduced *ex vivo* with a lentiviral vector (LV) encoding for the human *ARSA* gene, infused after intravenous myeloablative busulfan conditioning. Arsa-cel has shown favourable long-term outcomes in early-onset MLD; however, its risk-benefit profile in late-onset subtypes, in particular the Late Juvenile subtype (LJ), has not yet been investigated. This ongoing phase III trial (NCT04283227) is investigating the safety and efficacy of arsa-cel in pre-symptomatic or early-symptomatic (able to walk independently and without cognitive decline) LJ-MLD patients (expected or actual symptom onset between 7^{th} and 17^{th} birthday). We report preliminary engraftment and safety data from an *ad hoc* interim analysis.

As of March 2024, all 6 patients planned in the protocol were treated. Four patients were pre-symptomatic and 2 were early-symptomatic. Before enrolment in this study, one patient underwent allogeneic HSC transplant and experienced autologous reconstitution with absence of

donor chimerism, enabling eligibility for HSC-GT. Median age at GT was 10.4 years (range 2.7-15.5 years). Stem cell source was mobilized peripheral blood. Median yield was 32.67x10^6 CD34+cells/kg (range 26.54-36.27x10^6 CD34+cells/kg). After transduction, the infused drug product median dose was 23.1x10^6 CD34+cells/kg (range 16-28.94x10^6 CD34+cells/kg) with a median vector copy number (VCN)/cell of 3 (range 2-5). All patients received busulfan-based myeloablative conditioning with a final cumulative target area under the curve of 85 mg*h/L. After a median follow-up of 17.8 months (range 2.6 to 24.2 months) all patients are alive with no treatment-related serious adverse events. The type and nature of adverse events were consistent with the known safety profile of busulfan. There were no malignancies, no evidence of abnormal clonal expansion or replication-competent lentivirus, and no evidence of immune response against ARSA enzyme. All patients achieved normal hematological reconstitution and engraftment of transduced cells in line with previous experience in early-onset MLD. Median time to neutrophil and platelet engraftment was 34.0 days (range 26-42) and 28.5 days (range 25-50), respectively. One subject, the one previously treated with allo-transplant, experienced prolonged thrombocytopenia which resolved after a 5 month course of TPO-agonist. At 90 days post-treatment, in 5 patients with available data, median VCN in peripheral blood mononuclear cells (PBMCs) and CD15+ cells were 0.49 and 0.82 copies/cell, respectively; the percentage of transduced bone marrow progenitors ranged between 23.81 and 81.25%. At 6 months post-treatment, ARSA activity was restored to supranormal levels in PBMC (data available in 5 patients) and up to normal levels in cerebrospinal fluid (CNS pharmacodynamic efficacy measure, available in 4 patients).

All patients treated while pre-symptomatic and one while early-symptomatic remained neurologically stable. One patient treated while early-symptomatic showed disease progression early after gene therapy with stabilization by 12 months post-treatment.

This analysis demonstrates that the preliminary results on safety of this LV based HSC GT strategy and pharmacodynamics efficacy in LJ-MLD were similar to those reported in early-onset MLD. Longer follow-up is needed to assess clinical efficacy.

OR100

Phase 1/2 dose-finding study to evaluate systemic administration of an AAV9-based gene therapy for peripheral manifestations of Gaucher disease: The Proceed Trial

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Gaucher disease (GD) is caused by bi-allelic *GBA1* mutations, resulting in glucocerebrosidase (GCase) deficiency. GD typically presents in early adulthood with hepatosplenomegaly, anaemia, and thrombocytopenia. Patients may also develop osteopenia, skeletal abnormalities, and pulmonary infiltrates, and may have a higher risk for developing Parkinson's disease. Enzyme replacement therapy (ERT) and substrate reduction therapy (SRT) may not resolve all peripheral

symptoms, and in the case of ERT is highly burdensome. A gene therapy approach in which a healthy gene is transferred to certain cells to constitutively and persistently express a deficient protein may offer a convenient, long-term therapeutic solution. We describe a Phase 1/2 trial testing systemic delivery of an rAAV9-GBA1 gene therapy construct to treat the peripheral manifestations of GD.

PROCEED is an open-label, Phase 1/2, multicentre dose-finding trial to evaluate the safety and efficacy of single-administration LY3884961 in individuals diagnosed with peripheral manifestations of GD.

Up to 15 patients aged 18-65 with bi-allelic *GBA1* mutations will receive LY3884961 via a single intravenous injection in one of three sequential escalating doses. The primary objective is to evaluate the safety and tolerability of LY3884961. Secondary and exploratory objectives include time from ERT/SRT discontinuation and time to ERT/SRT re-initiation, if clinically necessary, and changes from baseline in platelet count, spleen and liver volumes, bone marrow involvement, bone mineral density, lung function and pharmacodynamic changes in biomarkers, including GCase activity and glycolipids.

The PROCEED trial (NCT05487599) will assess whether a single intravenous administration of LY3884961 can safely provide clinical stability or improvement on peripheral measures of GD, after withdrawal of ERT/SRT. Initial clinical and biomarker data suggest LY3884961 to be safe, well-tolerated, and has the potential to be a one-time treatment for Gaucher disease Type 1.

Enrolment in the PROCEED trial is ongoing; an update will be provided.

OR101

Gene therapy for CLN3, Juvenile neuronal ceroid lipofuscinosis, a promising therapy

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Mutations in the *CLN3* gene cause juvenile NCL or Batten's disease, a severe neurodegenerative disorder leading to blindness, motor impairment, dementia, and intractable epilepsy. We developed an adeno-associated virus serotype 9 based gene therapy for treatment of this disease. We provide an update on the preliminary results of the first in human intrathecal gene therapy clinical trial in patients with mutations of the CLN3 gene. The human *CLN3* cDNA was transferred using self-complementary AAV9, driving expression under control of a truncated Methyl CpG binding protein 2 promoter (P546). Treatment was well tolerated in the first three human subjects treated with $6x10^{13}$ viral genomes (vg) and one subject treated with $1.2x10^{14}$ vg. The 3 low-dose subjects included 2 boys and 1 girl (Subjects 1-3) aged 114, 105, and 71 months at enrolment, respectively. The high-dose subject (Subject 4) was a boy aged 120 months.

Most treatment-emergent adverse events (AEs) were mild or moderate and unrelated to scAAV9.P546.CLN3. One subject experienced a serious adverse event with an elevated alanine aminotransferase level) that resolved, while Subject 3 experienced serious AE of seizure that was disease related. Anti-AAV9 antibody titers were low in all subjects.

Efficacy results at 36 months post gene therapy show lack of disease progression in 3 of 4 children compared to natural history which show a gain of +2.86 points/year (approximately +8.58 points in 3 years) on the UBDRS physical impairment scale. In contrast, our patient cohort

UBDRS physical impairment scores at 36 months were -2, -1, +4, and +1 respectively. The youngest patient is seizure free at the age of 11 and learned to read indicating slowed disease progression. The only patient who received high dose gene transfer has been seizure free for two years on a single antiepileptic medication. Our results show demonstrate safety and suggest efficacy in 3 out of 4 patients warranting further evaluation in a larger patient cohort.

OR102

REKLAIM, a Phase I/II clinical trial using a novel immune modulation strategy for systemic administration of FBX-101 (AAVrh10.GALC) after Umbilical Cord Blood Transplantation for the treatment of Infantile Krabbe Disease

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IKD is a fatal neurodegenerative disorder due to galactocerebrosidase (GALC) deficiency that results in psychosine toxicity to myelinating cells in the brain and peripheral nervous system. If untreated, death occurs at a median of 2 years. Currently,the standard of care for pre-symptomatic neonates is treatment with UCBT that halts the brain demyelination, but motor function continues to decline due to progressive peripheral neuropathy. Recently newborn screening for Krabbe was recommended by the US Advisory Committee for Heritable Disorders for Newborns and Children to be included in the Recommended Uniform Screening Panel substantiating the need to treat this devastating disorder.

REKLAIM is a novel intravenous AAVrh10.GALC gene therapy administered during myeloablation or immune suppression both of which are necessary for the treatment of umbilical cord blood transplantation (UCBT) for infantile and Late Infantile Krabbedisease (IKD, LIKD). We report the results of the first 5 subjects with IKD treated with a low dose of intravenous FBX-101 (1.6 x1013 gc/kg). We hypothesized that FBX-101 administered during myeloablation will override the antibody response to the vector's capsid and transgene since the UCBT provides a healthy but immature donor immune system that does not recognize GALC as an antigen.

REKLAIM is a Phase I/II dose-escalating intravenous gene therapy to evaluate safety and efficacy of FBX-101 administered systemically more than 21 days after UCBT infusion and during myeloablation or later when the subject is immune suppressed. For those treated during immune suppression, the regime is individually evaluated by the PI and a proposal submitted, evaluated and approved or modified by an independent committee of experts. The baseline protocol includes Rituximab, Serolimus and Prednisolone that is implemented according to the subject needs.

FBX-101 in the 5 patients receiving the low dose was well tolerated, with no treatment-related serious adverse events during a follow up ranging from 6 to 24 months. No antibodies to the transgene developed in any of the subjects. In the two subjects treated during myeloablation, there were no antibodies to AAV10. Plasma and CSF GALC significantly increased, psychosine dropped below the level of detection and subjects white matter growth had a normal trajectory,

peripheral nerve conduction velocity in 4 of 5 measures at the last evaluation reached the range of normal and 1 was borderline normal.

Gross motor skills as measured by the PDMS-II are normal. For the three subjects treated during immune suppression, total antibodies to AAV10 were increased after infusion with no signs of humoral or cellular toxicity. GALC increased to a lesser extend than in the myeloablated group and psychosine decreased but have not yet normalized. All subjects improved in gross motor function. In summary, FBX-101 after UCBT leverages the myeloablation and immune suppression after UCBT, resulting in efficient AAV transduction and providing increased GALC enzyme and decreased psychosine that results in supporting brain and peripheral myelination and gross motor development. All 5 patients are walking. The DSMB has approved to move into the high dose cohort.

OR103

mRNA-replacement therapy for Glycogen Storage Disease type 1b

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Glycogen storage disease type 1b (GSD1b) is an inherited metabolic disorder due to deficiency of glucose-6-phosphate transporter (G6PT). GSD1b presents in the first months of life with hypoglycemia, growth retardation, osteoporosis and long-term risk of renal failure and liver cancer. In contrast to Glycogen storage disease type 1a, GSD1b patients also show neutropenia associated with recurrent infections and inflammatory bowel disease. Current treatments for GSD1b include management of hypoglycemia by diet and therapy with granulocyte colony stimulating factor (G-CSF) or the sodium–glucose cotransporter 2 (SGLT2) inhibitor for correction of the neutropenia. However, available treatments are unsatisfactory, and the disease morbidity remains high. Therefore, the development of more effective therapies is highly needed. Therapy with mRNA encapsulated into lipid nanoparticles (LNPs) is emerging as an attractive platform for in vivo gene replacement therapy in several inherited metabolic liver diseases. To investigate the efficacy of mRNA replacement therapy in GSD1b, we generated G6pt$^{-/-}$ mice that were found to have increased perinatal mortality and abundant liver glycogen storage. Intravenous (IV) injections of mRNA encoding G6PT encapsulated into lipid nanoparticles (LNP-G6PT mRNA) performed shortly after birth significantly increased survival of newborn G6pt$^{-/-}$ mice compared to standard glucose injections. Surviving adult G6pt$^{-/-}$ mice developed hypoglycemia during fasting that was corrected for at least one week by IV injections of LNP-G6PT mRNA. In conclusion, our study provides for the first proof of efficacy of LNP-G6PT mRNA in a preclinical mouse model of GSD1b and this study might pave the way towards the development of a clinical trial for GSD1b patients.

OR104

A safe Peptide-based, lipid-free, nanoparticle platform for mRNA delivery and gene editing in the liver: application to Factor VIII rescue in hemophilia A and selective editing of PCSK9 gene to durably lowers cholesterol in mice

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Although safe and efficient delivery of therapeutic mRNAs offers great promise, it remains challenging due to hepatic accumulation and dosed-limiting toxicity of the commonly used LNPs. To overcomes these issues we have developed a new peptide-based, lipid-free, delivery platform that can potently deliver functional mRNAs or gene editing machinery in the liver. The therapeutic potency of the platform was assessed by delivering functional mRNAs rescuing Factor VIII level in a Hemophilia A mouse model and selectively editing the *PCSK9* gene in mouse liver.

Pep-NP technology is based on short amphipathic peptides that form stable scalable nanoparticles able to efficiently package and deliver RNA to the target site. The chemistry of the peptide can be tuned in order to induce robust mRNA expression in specific organs. Peptides with a liver tropism and able to target the liver were selected. The peptide/mRNA nanoparticles are stable both in solution and as dry powder, with an average size ranging between 40-100 nm and a high encapsulation efficiency (>90%). We demonstrated that IV-administration of peptide/mRNA luciferase or peptide/Cas9mRNA:gRNA nanoparticles resulted in mRNA accumulation in the liver with either luciferase or CAS9 protein expression starting 6 hr after administration with an optimal expression at 24 hrs.

Hemophilia A is an X-linked bleeding disorder caused by the deficiency of coagulation Factor VIII. we showed that intravenous (IV) and subcutaneous (SQ) administrations of Pep/mRNAs-FVIII NPs in a Hemophilia A mouse model, using different dosing regimens (0.2 to 1.0 mg/Kg), results in a rapid pulse of FVIII protein expression and a recovery of the Factor VIII level in the blood. The FVIII protein expression and activity remained above therapeutic levels (i.e.>50% of reference) 20 days post injection and slowly declined thereafter. Repeated IV (0.2 mg/kg) and SQ (1.0 mg/kg) dosing of Pep/mRNAs-FVIII once every 3-weeks produced consistent expression of FVIII over time and maintained protein level and FVIII activity above clinically relevant threshold. Our study suggested that Pep/mRNAs-FVIII given IV or SC every 3 weeks can maintain therapeutic levels of FVIII-protein.

Atherosclerotic cardiovascular disease remains the leading cause of death worldwide. Hypercholesterolemia is a highly prevalent genetic disorder and associated to mutations in proprotein convertase subtilisin/kexin type 9 gene (PCSK9). Pep-NPs were used for the co-delivery of mRNACas9 and sgRNA targeting exon 1 of mouse *PCSK9*. We showed that a single IV-administration of Pep-NP-PCSK9 (0.2 mg/kg) resulted in ca.75 % of *PCSK9* gene knockdown in the liver. Pep-NP-PCSK9 treatment induced a stable reduction of both circulating PCSK9 plasma levels by ca. 80% and plasma cholesterol level by ca. 65%. These effects were maintained 4 months after a single-dose treatment and no off-target effects were observed at 2.0 mg/kg dose.

Peptide/mRNA nanoparticles are well tolerated. Cytokine, histology and liver enzyme profiling showed that repeated SQ and IV administrations did not induce liver toxicity, inflammation or chronic alteration. The peptide/mRNA, lipid-free nanoparticles appear to be a safe and effective

platform for tissue-specific delivery of functional RNA therapeutics including gene editing of target genes in the liver with potential clinical applications.

OR105

Alpharetrovirus-like particles for *in vitro* and *vivo* delivery of diagnostic and therapeutic RNAs

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Transient expression of certain transgenes can be useful for many applications, such as cell fate modification or genome engineering strategies. Recently, we developed chimeric alpharetrovirus-based particles, in which the retroviral packaging machinery was replaced with the packaging system of the MS2 bacteriophage. This modification allowed the specific packaging of different RNA classes devoid of any retroviral sequences within the same or into separate particles. We succeeded in transiently transferring receptors, cytoplasmic proteins as well as DNA modifying enzymes, including CRISPR/Cas9 designer nucleases to cell lines and primary cells.

In this study, we examined the *in vivo* functionality of these particles in terms of their transduction efficiency and immunogenicity and show first *in vitro* data on their use for CRISPR/Cas9-based adenine base editing. To show the *in vivo* applicability of our particles, we produced virus-like particles encoding for the firefly luciferase gene and delivered these to the liver of Balb/C mice as a target organ. In addition, we produced a second particle type that contains the CD47 self-protein in its lipid envelope as a strategy to prevent phagocytic uptake. Although VSVg displays a broad tropism, we pseudotyped both particle variants with VSVg, as it naturally favors the biodistribution to the liver and hepatocytes highly express the low density lipoprotein receptor, the main entry receptor of VSVg.

Strikingly, 24 h after tail vein injection of the virus-like particles, Balb/C mice showed strong luciferase expression in the liver, with superior expression levels induced by CD47-containing particles. Time course analyses of injected and living mice, revealed dose-dependent luciferase expression of up to four days, indicating the transient and controllable nature of this technology.

To assess immunogenicity and cytotoxicity, we measured the levels of 13 different inflammatory cytokines as well as liver enzymes in the blood of treated mice, with no significant elevations observed. To move one step further towards application in gene therapy, we produced all-in-one virus-like particles delivering the CRISPR/Cas9-based ABEmax base editor mRNA and a specific sgRNA to correct the murine C282Y mutation causing hereditary hemochromatosis. In reporter cells or isolated primary murine hepatocytes from the hemochromatosis mouse model, we achieved an A>G correction of up to 66% or 29%, respectively.

In summary, our study demonstrates that chimeric alpharetrovirus-based particles are a promising technology for *in vivo* gene therapy applications and in particular for genome editing strategies.

OR106

DLVR-M: a novel fully humanized particle for the efficient *in vivo* delivery of large gene-editing cargos to human cells

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An urgent need exists for novel delivery modalities compatible with *in vivo* applications to fully realize the therapeutic potential of gene editing technologies. Virus-like particles (VLPs) provide an alternative to LNPs or AAVs, offering advantages including: 1. delivery of a wide range of larger cargos, 2. protein cargos with shorter half-lives than nucleic acids, 3. lack of potentially integrative DNA, and 4. re-targetability. However, VLPs still harbor multiple viral proteins, such as GAG, POL, and ENV, which may elicit immunogenicity, complicate manufacturing, and constrain the size of these particles, therefore limiting their reach upon systemic delivery. An ideal platform should maintain the VLP cargo capacity, without harboring viral components or being limited by particle size constraints.

We here present the design, optimization, and *in vitro* and *in vivo* testing of DLVR-M, a novel delivery platform. DLVR-M particles do not contain the viral GAG and POL proteins found in VLPs, but instead utilize a compact human protein-derived architecture that simplifies manufacturing. We optimized DLVR-M to efficiently and non-covalently anchor cargos of interest directly to the inner leaflet plasma membrane of producer cells. We designed our DLVR-M particles to display ENV proteins that enable high transduction efficiency, a feature that distinguishes them from other native extracellular vesicles. We show that DLVR-M particles can be used to deliver a wide range of gene editing cargos including CRISPR and non-CRISPR nucleases, base editors, prime editors, and programmable transcription factors, inducing high frequency targeted edits into a wide variety of different cell types in culture, including primary human hepatocytes and primary human T cells. Importantly, we will show that DLVR-M particles can generate robust and efficient adenine base editing *in vivo* in mouse hepatocytes at the *PCSK9* gene locus (41% A>G average conversion resulting in 75% average serum reduction of PCSK9), at doses competitive with VLPs.

We will then describe preliminary data showing the development of completely humanized DLVR-M particles (huDLVR-M), in which the viral ENV is replaced with novel engineered human-derived fusogens. We will show that huDLVR-M particles pseudotyped with different envelopes can deliver CRISPR gene editing nucleases and base editors with high efficiency into a broad range of therapeutically relevant primary human cells including neurons, hepatocytes, and skeletal muscle cells. Notably, we report >80% editing at low doses of huDLVR-M in primary unstimulated hematopoietic stem and progenitor cells. We are currently testing huDLVR-M *in vivo* in humanized animal models. Lastly, addressing the current biodistribution limitations imposed by size constraints of viral-derived platforms, we will introduce nanoDLVR-M; humanized particles produced via a novel method with smaller diameters than conventional VLPs and improved cargo capacity and potency.

Overall, the DLVR-M and huDLVR-M platforms can deliver a wide range of diverse cargos to a variety of different cell types while simplifying manufacturing with a design free of virus-derived

components. This novel particle has the potential to revolutionize many research and therapeutics applications currently limited by the capabilities and characteristics of existing delivery modalities.

Poster Presentations

P0001

A multifaceted strategy for viral safety and high productivity in AAV processes

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The resurgence of *in-vivo* gene therapies is providing lifesaving options to patients with otherwise untreatable diseases, leading to first commercial products and to an increased demand for large amounts of high-quality adeno-associated virus (AAV) viral vectors. This is especially true for systemic gene therapies and those targeting specific tissues or organs where a higher vector dose is needed to achieve therapeutic effects. The insect cell based Baculovirus Expression Vector System (BEVS) based on baculovirus infections for production and Baculovirus Infected Insect Cell system (BIICs) based on infected cells for production are commonly used to produce AAV vectors in insect cells for large-scale production of AAV-based gene therapies. This system offers advantages such as high yields and the ability to produce high-quality AAV vectors suitable for high-dose therapeutic applications. *Spodoptera frugiperda* (Sf) cell lines are widely used as hosts for BEVS and BIIC. However, the majority of Sf9 and Sf21 cell lines contain a Sf-rhabdovirus which is considered a process contaminant and must be eliminated from the product. To improve the safety profile of the BEVS and BIIC production methods, we developed a high performing Sf-rhabdovirus-negative (Sf-RVN®) Platform, composed of an Sf9 rhabdovirus-free cell line with an optimized chemically defined medium (EX-CELL® CD Insect Cell Medium).

The identification and optimization of upstream critical process parameters represent a pivotal strategy for intensifying AAV vector production. In this study, we focused on the multiplicity of infection (MOI) and the ratio of components necessary for viral DNA replication and packaging of the gene of interest (GOI) for optimizing the efficiency of baculovirus-mediated AAV production. We achieved high titres using our Sf-RVN® insect cell line cultivated with our chemically defined medium, and we further increased AAV productivity and percent full capsids through post-infection media feed strategy.

P0002

Crossing the boundaries of AAV production: harnessing high salt concentrations to improve process efficiency

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Improving adeno-associated virus (AAV) production is of utmost importance in the field of gene therapy.

In this study, we confirmed previous approaches to boost AAV production using higher salt concentrations during the lysis process. Our research yielded impressive results, demonstrating that high levels of salt, such as 500mM, effectively enhance AAV process intensification by removing adhesive DNA from AAV capsids and reducing aggregation.

However, it should be noted that high salt concentrations exceeding 200mM hamper the activity of standard endonucleases, which are virtually inactive in a high-salt environment. To meet this challenge, we have developed a state-of-the-art enzyme, the salt-tolerant endonuclease Benzonase®, specifically designed to function optimally in high-salt conditions ranging from 500 to 1,000 mM. This remarkable enzyme efficiently digests DNA fragments of less than 10 base pairs, and remains fully compatible with commonly used detergents.

Incorporating 500mM salt into the lysis buffer produced remarkable results. Our experimental results indicate a substantial improvement in AAV titers of 20-30% (assessed by ELISA) and a remarkable improvement in viral transduction capacity by a factor of 10. These results provide valuable pointers for scaling up AAV production, resolving one of the major bottlenecks in the gene therapy industry: AAV production yield. The design of this new salt-tolerant IPEC GMP endonuclease also contributes to meeting the most stringent patient safety standards.

P0003

Advanced monitoring of AAV vector production by online capacitance spectroscopy

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AAV vector production is a complex process in which the cultivation of human embryonic kidney cells (HEK293) plays a critical role in the generation of high-quality viral vectors. Tracking the Viable Cell Concentration (VCC) during upstream AAV vector production is essential for process monitoring and for implementing actions to ensure optimal process management. The advent of inline capacitance probes has introduced a crucial Process Analytical Technology (PAT) tool for the real-time measurement of VCC, with improved measurement accuracy by application of frequency scanning and multivariate modelling tools. Here we present the development and application of a method for real-time online monitoring of VCC in HEK293-based cell cultures used for recombinant AAV vector production. In a first step, BioPAT® Viamass MU probes were used to record capacitance signals of individual 10L AAV-8 batches within a frequency range of 50 kHz – 20MHz. Based on scan data, an Orthogonal Partial Least Squares (OPLS®) model for accurate VCC prediction was developed in SIMCA® software. Subsequently, the obtained model was deployed online and VCC predictions were exposed into BioPAT® MFCS bioprocess control software, enabling real-time VCC monitoring in consecutive production batches and accurate estimation of the transfection time point. To the best of our knowledge, this is the first example of online deployment of a VCC predictive model for real-time monitoring of this parameter in HEK-cell based transient AAV vector production. The ability to monitor VCC continuously and predictively is a significant advancement in the field, offering the potential to revolutionize AAV

production by optimizing process events such as transfection timing, and thus enhancing viral yield and quality.

P0004

AAV process intensification: focus on upstream critical process parameters

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Adeno-associated virus (AAV) vectors have emerged as a promising platform for gene therapy due to their safety and long-term gene expression. However, the production of AAV vectors has historically faced challenges related to low process yields and high production costs. As the demand for AAV therapeutics increases, so does the need for high levels of infectious AAV particles to support this rapidly growing industry. One key area of AAV process intensification, especially with transient transfection process, lies in optimizing the critical process parameters in the upstream phase of AAV vector production. To maximize AAV titres across multiple HEK293 cell lines, media development for AAV production was performed with three HEK293 cell lines with different growth rates. We demonstrated that the media optimization steps enabled improvement to support rapid growth of all cell lines evaluated. Following media development, we have sought to optimize the transfection using polyethyleneimine (PEI) and, bioreactor parameters from microbioreactor scale to Mobius® 3L. Thanks to transfection optimization, we shown an improvement over 10x of the AAV titre. Finally, we optimized the feed strategy to further increase AAV productivity.

The identification and optimization of upstream critical process parameters represent a pivotal strategy for intensifying AAV vector production. Through the targeted optimization of chemically defined media performances across multiple HEK293 cell lines, of PEI-based transfection parameters, and the development of post-transfection feed strategy, we achieved substantial increases in AAV vector titres, thereby addressing a key bottleneck in the manufacturing process.

P0005

A sequential administration of adeno-associated virus-Thp1 and baculovirus-Thp1 as a vaccine could protect mice from *Helicobacter pylori* infection

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Diseases such as chronic gastritis, peptic ulcers, MALT lymphomas and stomach cancer are mainly caused by the bacterium *Helicobacter pylori (H. pylori)*, which infects more than 50% of the world's population (4.05 billion people), causing gastric-duodenal ulcers in 0.4-0.6 billion people (10-15%). This implies that potentially 40 million people (1%) will develop stomach cancer. Currently, therapies to eliminate *H. pylori*, which consist of the use of two or three antibiotics and a proton pump inhibitor, have declined in efficacy due to the emergence of antibiotic-resistant strains and the ability of the bacterium to adapt to adverse conditions. Notably, to date, there is no vaccine available to eradicate *H. pylori* infection and prevent the development of these potentially fatal gastric diseases. In the search for more effective strategies for vaccine development is the use of viral vectors, as they provide highly efficient gene transduction, can deliver genes in a highly specific manner to target cells, elicit strong immune responses directed against pathogen antigens and enhance cell-mediated immunity without the need for adjuvants. In this work we propose the development of a new generation vaccine to block *H. pylori* activity using adeno-associated viruses (AAVs) and baculoviruses as platforms. We previously reported the generation of the Bac-Thp1 baculovirus. In this work we cloned the same Thp1 transgene into the AAV genome (AAV-Thp1), consisting of nine epitopes selected from the literature or by bioinformatics (*in silico*); the epitopes were identified in *H. pylori* proteins: carbonic anhydrase, urease B, gamma-glutamyl transpeptidase, Lpp20, Cag7 and CagL. The viral vectors separately or in combination were administered as a vaccine in mice to determine the activation of the immune response and to identify the inhibition of infection upon challenge with *H. pylori*. Our initial results show that administration of three i.m. or i.g. doses of adeno-associated virus AAV-Thp1 in combination with an i.g. boost with baculovirus Bac-Thp1 generated a strong IgG antibody response specific for *H. pylori*. This antibody level increased after challenging the mice with the bacteria. We are identifying the effect of these vaccines at the level of mucosal response and protection from *H. pylori* infection and gastric damage. The vaccine based on the combination of AAV-Thp1 and Bac-Thp1 shows promise for controlling ulcers, gastritis and stomach cancer associated with *H. pylori* infection in humans.

P0006

AAV9-JGA: A novel vector for kidney-specific targeting of juxtaglomerular apparatus after systemic administration

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Natural AAV serotypes lack efficient and specific kidney cell targeting. We addressed this challenge by developing an in vivo selection protocol for kidney-specific AAV vectors. A liver de-targeting display peptide library was screened, leading to the identification of AAV9-JGA. This novel vector efficiently and specifically transduced a subset of the distal tubules at the juxtaglomerular apparatus (JGA) after systemic administration. The renal tubules are considered inaccessible to AAV, because circulating AAV cannot cross the glomerular filtration barrier due to size restrictions. We traced AAV9-JGA after systemic administration using electron microscopy. Surprisingly, we discovered that AAV9-JGA bypassed the glomerular filtration barrier and reached the distal tubule via a previously unknown route - the tubule-afferent arteriole contact. As the JGA is one of the most important structures in the kidney that regulates the hemodynamics

through tubuloglomerular feedback, AAV9-JGA-mediated delivery of a diphtheria toxin receptor allows us to deplete these specialized cells and thereby investigate their physiological function. In conclusion, this study provides new insights into the understanding of AAV transduction in the kidney. We have discovered a novel AAV targeting a subset of the distal tubule at the JGA and uncovered a natural route of AAV transduction in renal tubules after systemic administration. Future work will elucidate the function of these specialized cells and explore the potential of AAV9-JGA for kidney-targeted gene therapy.

P0007

Step by step development of a cell-based potency assay for the gene therapy product G3MDYF/GNT0004 (rAAV8 human Microdystrophin)

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The potency (or biological activity) is a critical quality feature of biological medicinal products, as gene therapy vectors. A potency assay should show the Mechanism of Action (MoA) of the product and ideally reflect the clinical response. Considering the complexity of gene therapy products as viral vectors, for the development of the potency test of G3MDYF/GNT0004 (rAAV8 human Microdystrophin) we proceed step by step, demonstrating in each step a biological achievement of the vector necessary to carry out the MoA. Each step represents a cell-based assay performed on human muscle cells (derived from Duchenne Muscular Dystrophy patient) transduced with AAV8-Microdystrophin (AAV8-hMD1) and differentiated for four days. In the first step we detected the transgene's expression of the AAV8-hMD1 at the mRNA level by quantitative reverse transcription polymerase chain reaction (RT-qPCR). We evaluated the relative potency which refers to the ability of the test batch to produce the desired response compared to a development standard batch, when tested under the same conditions. We estimated a good intermediate precision of the method, as the CV of the relative potency score was below 20%. The second step is based on the detection of the hMD1 protein by Simple Western. We estimated the intermediate precision of the method as for the first step and we determined the robustness of the method verifying that linearity, precision and relative potency score were not affected by a small modification of a parameter, in our case the temperature of protein denaturation. Since to exploit its function hMD1 must reach the membrane, in the third step we characterized the localization of the hMD1 protein by two different techniques: immunostaining to verify the colocalization between hMD1 and the dystrophin-associated protein complex (DAPC) proteins and Simple Western after subcellular fractionation to detect hMD1 protein in the membrane fraction and measure the relative potency in a dose dependent manner. We have shown the combination of different techniques to develop a robust assay for G3MDYF/GNT0004 (rAAV8 human Microdystrophin), that is fundamental for comparability studies, process validation and for stability testing.

P0008

Establishment of a stable producer cell line capable of high-titer production of therapeutically relevant Adeno-Associated Virus (AAV) vectors

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Adeno-associated virus (AAV) vector-mediated gene delivery is increasingly becoming routine in the field of gene therapy. To satisfy the escalating demand for materials, the establishment of stable cell lines for the production of AAV vectors is being recognized as a significant development within the industrial and scientific communities. The primary benefits of this approach include the ability to scale up production and achieve high consistency, along with a reduction in production costs when contrasted with the triple-transfection process.

In this particular case study, we describe the generation of a stable HEK293-based producer cell line with high-yield capacity for AAV vector production, incorporating a non-clinical variant of alpha-Glucosidase (GAA) as the transgene and a genetically engineered capsid. This endeavor was a collaborative effort involving Roche, Spark Therapeutics, Inc., and Cytiva (formerly Cevec Pharmaceuticals). The central objective of this proof-of-concept study was to validate the feasibility and efficiency of the ELEVECTA™ stable producer cell lines in the production of therapeutic AAV vectors.

On the poster we will elucidate the procedures and results involved in the stepwise generation of a mixed producer cell pool and the subsequent isolation of single-cell clones, as well as the characterization of these pools and clones using different production systems, such as 96 deep-well plates or the Ambr15 micro-bioreactor system. A stringent screening funnel will be presented that allowed for a step-wise constriction in clone numbers. Additionally, we will share the outcomes of a stability assessment that models the cultivation time starting from a single vial of frozen cells to a large scale bioreactor. Finally, we will show the outcome of a mid-scale bioreactor production with two selected clones and highlight the increase of productivity over production scales and degree of system regulation during the ELEVECTA™ process.

P0009

Minimizing DNA impurities and host cell DNA packaging during the production of recombinant adeno-associated virus (rAAV)

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Recombinant adeno-associated viral vectors (rAAV) are medicinal modalities extensively used in gene therapies to introduce a genetic modification supporting a therapeutic effect. It is standard in the pharmaceutical industry to establish the safety and efficacy of new products during clinical development prior to marketing authorization by the competent authorities. The primary goal of

an early-stage clinical trial is to establish the safety of a product. Product developers should therefore have a strong focus on product safety during the initial steps of development. Residual DNA impurities coming from the production host cells represent a safety concern since host cell DNA has been associated with risks of oncogenesis (eg: active oncogene), and infection (eg: retrovirus). Monitoring the amount of residual host cell DNA is part of the control strategy, and the process of production should be designed to limit the amount of host cell DNA. Minimizing DNA impurities can be particularly challenging for rAAV products with a high administration dose. We examine here some levers to reduce the safety risks associated with DNA impurities in rAAV products.

P0010

Drug product formulation fit approach to accelerate AAV drug product development to IND

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Formulation plays a crucial role in maintaining the stability of a drug product (DP) over its shelf life. However, the standard formulation development approach is often time-consuming and labor-intensive due to extensive testing required for different excipient combinations and conditions. To tackle this challenge and reduce costs, we leverage standard in-house formulation buffers (FBs) developed for adeno-associated virus (AAV) DPs to meet the requirements of new target AAV product profiles. Leveraging is based on an existing in-house formulation buffers library that over the years was built for the uniQure AAV products by using a standard formulation development approach. Compared to the standard formulation development timeline of one year, the formulation fit (FF) approach significantly reduces the time needed to decide on the formulation to approximately 10 weeks. Presented here is a case study on FF.

In this study, we detail the design, execution, and outcomes of the FF study conducted on one of uniQure's AAV DP. The primary objective was to assess the stability of the AAV DP in three different in-house FBs under various stress conditions. FB selection was based on the DP's administration route, while stress conditions mimicked real-life manufacturing and handling processes, including extreme conditions. Subsequently, we evaluated DP stability and compared it across different formulations based on aggregation profile and vector genome leakage. Among the three FBs tested, one formulation outperformed the others. The DP remained stable through freeze-thaw stress and up to one month of storage at 25°C, showing no visible particle formation, minimal aggregation, and DNA release.

Overall, this FF formulation fit study not only proved effective but also demonstrated its time-efficiency and cost-effectiveness compared to the standard formulation development approach and it is currently applied for uniQure’s platform-based AAV drug products development.

P0011

In vivo engineering of T cells using the evolved AAV variant Ark313

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Genetic engineering of T cells in mouse models allows for the interrogation of immune mechanisms and pathways. However, generating T cells with specific genetic changes in mice can be time and labor intensive, requiring either embryonic manipulation or *ex vivo* engineering prior to host immune conditioning and engraftment. To better study and manipulate T cell biology in mouse models for immunotherapy applications, we previously evolved an AAV capsid named Ark313 that efficiently transduces mouse T cells for *ex vivo* engineering. Here, we show that Ark313 enables potent and targeted gene transfer to mouse T cells *in vivo*. We demonstrate that delivery of transiently expressed genes is feasible using Ark313 and that this serotype vastly outperforms natural serotypes *in vivo*. With a single intravenous injection in the Ai9 fluorescent reporter mouse model, a Cre recombinase-expressing Ark313 can achieve permanent genetic changes in ~25% of T cells *in vivo* and displays reduced liver targeting relative to parental AAV6. Furthermore, Ark313 enables *in vivo* gene editing in both tissue-resident and splenic T cells, and validation of immunotherapy targets *in vivo* such as *Regnase-1* in a solid tumor model. Finally, as performing gene targeting *in vivo* in primary cells remains a challenge in the field, we demonstrate that Ark313 can deliver large DNA donor templates to T cells *in vivo* and integrate transgenes in both $CD4^+$ and $CD8^+$ T cells, including naïve T cells. Efficient targeting of mouse T cells *in vivo* holds great promise for the interrogation of T cell biology as well as studies for immunotherapies.

P0012

Development of a full capsid enrichment polishing step in AAV8 purification process with CIMmultus® PrimaT® monolithic column

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Gene therapies treatments based on AAV vectors are promising for rare diseases. However, while producing AAV vectors for these gene therapies, empty capsids are also produced in a certain amount. Clinical effect of empty capsids is unclear. Empty capsids are not the intended therapeutic product and could include a toxicological effect. Thus, process development teams need to reduce their presence. This objective could be achieved by designing new capsids and transgenes, by changing cell culture conditions or by implementing a polishing step on current purification process. The latter approach is evaluated using the CIMmultus® PrimaT® monolithic column developed by Sartorius. Parameters including viral loading, residence time, conductivity of loading and elution are investigated. Based on the generated data, optimal conditions are

determined to enrich the product in full capsids while maintained an acceptable viral genome yield. These conditions are subsequently applied up to 200 L scale. Results obtained confirm the performance, repeatability, and scalability of CIMmultus® PrimaT® monolithic column to remove empty capsids and some product-related impurities.

P0013

Evaluation of IdeS efficiency to reduce high titer of NAb and allow vector redosing in New Zealand White Rabbit

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The clinical successes and the approval of gene therapy drugs based on Adeno-Associated Virus (AAV) vectors support the potential of AAV-based gene therapies for the treatment of both rare and frequent diseases. However, immune responses directed against the AAV components are a major limitation to gene transfer, as they can prevent tissue transduction and may have an impact on safety and durability of gene transfer. Pre-existing anti-AAV neutralizing antibodies (NAb) are commonly found in humans, and following vector administration they are produced at high titers, persisting for several years, and preventing AAV vector re-administration.

Recently, IdeS, an endopeptidase secreted by Streptococcus Pyogenes able to cleave IgG, has shown in several animal models a high potential to reduce anti-AAV NAb, resulting in increased liver transduction and transgene expression.

Here, we investigated the potential of IdeS for vector re-administration in the context of extremely high anti-AAV NAb titers. The use of New Zealand White (NZW) rabbits as animal model was based on 1) IdeS cleaves rabbit IgG with similar if not superior efficacy than human IgG; 2) NZW rabbits tends to have very high humoral responses against antigens in general. To induce humoral response against AAV8 capsid, animals were first injected with an AAV8 vector expressing a reporter transgene. Two months after this first injection, they received IdeS before the re-administration of an AAV8 vector expressing a second reporter transgene. As expected, we observed a robust anti-capsid humoral response for all animals which was stabilized 2 months following AAV vector pre-dosing. In rabbits treated with IdeS, we observed a reduction of up to 98% of anti-AAV8 IgG compared to untreated animals with a rebound of these antibodies occurring five days after treatment. Nevertheless, a residual neutralizing activity was observed in IdeS-treated animals resulting to decreased transgene expression after vector redosing. To conclude, our results support the effective cleavage of anti-AAV8 IgG by IdeS. However, residual neutralizing activity, whose quantity is proportional to the initial concentration, could have a strong impact on gene transfer especially in patients having high levels of pre-existing antibodies or in the context of AAV redosing. In conclusion, our results support the extreme efficacy of IdeS for the cleavage of IgG at titers observed in the human population naturally exposed to AAVs. However, by showing that in the context of extremely high NAb titers, IdeS could not completely ablate the anti-AAV8 humoral response, we provide a strong rationale for the development of new endopeptidases with improved activity to achieve AAV vector re-administration.

P0014

Optimized biodistribution for CNS gene therapy

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The fundamental principle of gene therapy involves delivering functional genes or genetic material into target cells to compensate for defective genes, suppress harmful gene expression, or introduce novel therapeutic pathways. Recombinant Adeno-Associated Virus (AAV)-derived viral gene transfer vectors have become the most widely used vector systems in the human clinic for in vivo gene therapy strategies.

The pre-clinical data required by regulatory agencies before these gene therapy products can be used in humans is based on the consideration that any new construct must undergo a full evaluation, including a biodistribution study in animals.

Biodistribution studies are defined as the distribution, persistence, and clearance of a gene therapy product in vivo from the site of administration to the target as well as non-target tissues. It is important to define standard biodistribution parameters for these studies using AAVs of different serotypes, because currently there are no standards, and the vast majority of studies cannot be compared with each other. Ideally, these studies will be carried out using a quality assurance system to ensure traceability and validation of the data obtained.

This poster presents an optimized protocol from DNA extraction, to standardized approaches for quantitative real-time polymerase chain reaction (qPCR) with a unique reference gene used for normalization in a variety of tissues obtained from Mouse, NHP and Dog.

P0015

Evolution of a High-Producing, Modular Upstream Platform for AAV Manufacturing

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MeiraGTx is continuously developing its adeno-associated viral vector (AAV) upstream manufacturing platform to maximize yield and minimize the generation of non-therapeutic AAV particles and other process-related contaminants. MeiraGTx's upstream AAV production process is currently using a high-producing clonal HEK293 suspension cell line, an optimized triple plasmid system and a control strategy of key transfection parameters during scaling up. The work presented here shows the evolution of MeiraGTx's upstream manufacturing platform, which we were able to optimize and modulate through the choice of transfection reagents, AAV production enhancers, and transfection parameters, in fed-batch and perfusion culture mode. Over a three-year period, we have achieved process optimizations yielding AAV titers up to $1x10^{12}$ VG/mL at harvest and > 40% full capsids prior to polishing purification steps. Product quality attributes such as encapsidated residual plasmid DNA and host cell DNA are demonstrated to be controllable and maintained to satisfactory levels for patient safety through the combination of transfection reagent, small molecule enhancer and transfection mix formulation parameters. Operating a perfusion-based process has also increased volumetric VG yield by approximately 40% and

reduced plasmid DNA usage by at least 25%, without compromising on AAV productivity and product quality, demonstrating additional cost savings

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P0016

Proof of concept for the discovery of novel AAV capsids using a BRAIN-X insect cell-based library and rapid screening approach

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Developing novel adeno-associated virus (AAV) capsids through capsid engineering is crucial for enhancing gene therapy efficacy and safety. Traditional capsid engineering methods, relying on vector production in mammalian cells, pose challenges such as the requirement of high DNA quantities transfected per cell, leading to high cross-packaging level. Additionally, mammalian-based AAV capsids often require further adaptation for proper expression in the insect cell production platform. Here, we introduce BRAIN-X: a novel approach utilizing insect cells, which reduces the amount of DNA transfected per cell while maintaining high AAV capsid library titers. AAV capsid libraries constructed using the proprietary BRAIN-X methodology yielded deep and diverse libraries with minimized cross-packaging features. Concurrently, the BRAIN-X capsid library has been iterated in combination with next-generation sequencing (NGS) analysis and receptor-based *in vitro* evolution assays to discover novel human blood-brain barrier (BBB)-crossing AAV capsids. With only two cycles of *in vitro* evolution assisted by NGS enrichment analysis followed by rapid receptor-based *in vitro* assays, several human BBB-crossing capsid leads with putative consensus sequences have been identified. This study highlights the value of the BRAIN-X capsid engineering and screening methodology for the rapid development and deployment of novel capsids essential for advancements in gene therapy.

P0017

Automated plasmid purification can reduce manual labour and increase titres in AAV production

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An increasing number of adeno-associated virus (AAV)-based therapies are receiving approval for the treatment of genetic diseases, and the number in development are steadily increasing. However, high costs are hindering drug discovery, clinical development, and accessibility, and preparation of plasmid DNA has been identified as a bottleneck in both large- and small-scale AAV production. We have compared two systems for plasmid DNA purification and their use in AAV development and small-scale production: a golden standard commercial bench-top manual plasmid purification kit and a novel automated plasmid purification system. The three plasmids

required for AAV production was purified in Giga scale on each system. After purification followed evaluation of plasmid quality and preparation time, and the plasmids were used to produce AAV9 in two different HEK293-based production systems.

The automated plasmid purification resulted in 95% reduction of hands-on manual labour time compared to the manual kit, with 50% reduction of total process time. Applying the plasmids from the two purification systems in small-scale AAV9 production yielded similar AAV titres using PEI-based transfection in HEK293F cells. Surprisingly, using the AAV-MAX production system, the automatically purified plasmids produced 14-fold higher titres compared to the manually purified plasmids (3.2E+11 vg/ml and 2.3E+10 vg/ml respectively). Endotoxin removal of the plasmids prior to production further increased the titres to >7E+11 vg/ml. Thus, plasmids derived from the same bacterial culture but purified on two different plasmid purification systems can have major impacts on AAV titres and working hours required, depending on the transfection system used.

P0018

A simplified AAV infectivity assay enhances efficiency and throughput

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Adeno-associated virus (AAV) vectors are widely used in gene therapy, necessitating reliable and efficient assays to determine their infectivity. Traditional methods, such as the TCID50 assay, require extensive labor and resources, typically involving the use of two 96-well plates for a single sample. Here, we present a novel technique for assessing AAV infectivity that significantly reduces the workload and increases throughput. Our new assay method allows the simultaneous measurement of up to eight samples on a single 96-well plate. Although it does not provide an absolute TCID50 value, it offers a robust relative infectivity measure that is highly useful during process development. This assay enables researchers to quickly determine whether the infectivity of the virus is affected by specific treatments or steps in the production process.

P0019

Capsid engineering of novel AAV variants enabling gene delivery in choroid

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Age-related macular degeneration (AMD) is a common degenerative disease affecting the central retina (macula). A key feature is the formation of new choroidal blood vessels growing into and damaging the central retina. Implementation of anti-angiogenic drugs like aflibercept or brolucizumab has improved the prognosis of neovascular AMD. However, around 15% of patients do not respond to these treatments, even if the treatments are effective against neovascularization, they cannot halt retinal degeneration. In addition, the treatments have to be repeated every one to two months, which is a considerable burden for patients. A potential

curative solution might be gene therapy with adeno-associated virus (AAV) vectors targeting choroid to counteract neovascularization. However, successful gene transfer to choroidal vessels remains challenging. In this project, we engineered variants based on AAV1, the AAV serotype with the highest reported efficiency in endothelial cells transduction *in vivo*. The engineered AAV1 variants carry the peptide insertions first described in AAV2.GL and AAV2.NN, previously identified AAV2 variants with enhanced transduction efficiency for retinal cells. We used human umbilical vein endothelial cells (HUVEC) as a screening platform to evaluate CMV-mediated GFP transgene expression of different engineered AAV capsids. Subsequently, we assessed these vectors on mouse primary choroidal endothelial cells (ECs) and choroidal sprouting culture to evaluate cell type specificity. Subretinal injections were also performed to further confirm the transduction efficiency of novel capsids in *vivo*. In addition, human retina and choroid/RPE explants, as well as human retinal organoids were cultivated to evaluate the efficacy and selectivity of novel AAV1-based vectors. These novel vectors can robustly transduce primary mouse choroidal ECs (transduction efficiency in the range of 60% and higher), in contrast to parental AAV1 or other naturally occurring serotypes. Efficiency of gene transfer *in vivo* in mouse choroid and *ex vivo* in human retina and choroid/RPE explants, as well as human retinal organoid showed promising transduction efficiency and high specificity in targeting choroid. Currently, a gene editing approach based on the modified AAV1 vectors, targeting primary choroidal ECs is explored as a potential anti-angiogenic gene therapy. Our study identified that new AAV1-based capsid variants can transduce choroid efficiently, which can be confirmed by *in vitro*, *in vivo*, and human *ex vivo* culture. Our results showed that novel AAV1-based capsids hold high potential to be a valuable tool for the management of AMD in the future.

P0020

AAV-based evaluation of novel *in silico* promoters to drive expression in rod photoreceptors

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Overall, rods outnumber cones by a ratio of 1:20 or greater in the retina. Defects are common in rods and lead to various ocular diseases. To date, there are only a small number of rod-specific promoters. Therefore, we sought to design and test novel promoters to drive expression specifically and at high expression levels in rods.

Using an AI-assisted promoter engineering approach, ten novel promoter sequences were initially designed, cloned into an adeno-associated virus (AAV) backbone carrying eGFP and finally packaged into AAV5 or AAV7m8. In addition, AAV5 and AAV7m8 vectors were produced carrying the commonly used rhodopsin kinase (RK) promoter and eGFP. Following the initial screen, five second-generation, improved promoter sequences were designed combining elements from preferred performers of the first screen and again packaged into AAV5 and AAV7m8 for additional analysis.

To assess promoter activity in the murine retina, wild-type mice received subretinal injections with the AAV5 vectors. Four weeks post vector administration, eyes were harvested for immunohistochemical analysis and qPCR expression analysis to determine specificity and expression levels, respectively. In parallel, the AAV7m8 vectors were used to assess promoter activity in human pluripotent stem cell (hPSC)-derived retinal organoids. Three weeks post-

transduction organoids were fixed and dissociated into single cells for FACS analysis or cryosectioned for immunohistochemistry. Sections were stained with markers of rod and cone photoreceptors and quantitatively assessed for eGFP co-expression.

Lead candidates were identified on the basis of promoter specificity to rod photoreceptors, determined by immunohistochemistry, and promoter strength, measuring expression level using qPCR or signal intensity using FACS.

While multiple cone-specific promoters have been engineered, there is a lack of strong rod-specific promoters that could accelerate the clinical development of gene therapies to treat rod or rod-cone disorders as well as optogenetic applications.

P0021

Gene therapy as a potential disease-modifying approach in creatine transporter deficiency

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Creatine Transporter Deficiency (CTD) is a X-linked neurodevelopmental disorder characterized by cerebral creatine (Cr) deficiency, intellectual disability, seizures and autistic-like behavior. To investigate the potential for gene therapy as a disease-modifying treatment for CTD, we developed an adeno-associated 9 viral vector (AAV9) carrying a human *SLC6A8* transgene (encoding the creatine transporter, CrT) under the control of a ubiquitous promoter. The AAV9-*SLC6A8* vector was administered to male neonatal (postnatal day 1) CrT knockout (KO) mice and wild-type (WT) littermates via intracerebroventricular injection. Three weeks post-injection, western blot and immunofluorescence analysis revealed high expression and widespread distribution of the transgene into the brain, along with a significant increase in cerebral Cr levels measured by gas chromatography/mass spectrometry (GC/MS), thus confirming that the exogenous CrT was functional. The restoration of CrT led to the rescue of functional hypoconnectivity in KO animals and the improvement of autistic-like stereotyped behavior. However, this treatment did not improve cognitive function in KO mice and resulted in a deterioration of mnemonic performance in treated WT mice. Overall, our findings support the potential for gene therapy for the treatment of CTD, highlight the need for improved construct design to maximize therapeutic benefits and reduce safety risks. Ongoing efforts aim to develop cassettes that achieve more controlled levels and patterns of *SLC6A8* expression.

P0022

Effect of empty AAV capsids on biodistribution and immunogenicity

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Empty viral capsids are an inevitable byproduct of AAV production. The percentage of full capsids is an essential quality control parameter for any AAV lot, especially for in vivo studies and clinical/ commercial applications. There is a lack of data in the field by which to base numerical recommendations for cut-off criteria on the percent full capsid requirements for various AAV applications. To date, most recommendations to reduce empty viral particles have been largely driven by immunosafety concerns, with higher overall viral particles associated with robust anti-capsid immune response. The potential implications of empty AAV particles on genome biodistribution, and thus on potency, remain poorly understood.

In this study, the effect of increasing amounts of empty AAV viral particles on biodistribution in mice was investigated. Two different serotypes and routes of administration were used; AAV8 IV to assess systemic biodistribution, and AAV9 intracerebral ventricular (ICV) to assess central nervous system biodistribution. Both vector payloads consisted of factor IX (FIX) driven by the ubiquitous CMV promoter. Each group (n = 6) received a consistent vector genome dose of 5E13 vg/kg (IV) or 5E12 vg/kg (ICV), with increasing amounts of empty vector particles. One group per RoA received the maximum percent full from the AAV-FIX stocks, and subsequent groups received doses with 2-fold serial dilutions with the corresponding empty capsid stocks. The final percent full dilutions were as follows: 89%, 44%, 22% and 11% full. Blood samples were taken weekly, and tissues were collected at 28 days post-AAV administration.

Biodistribution was assessed by dPCR, and FIX expression was assessed by ELISA. Although subtle differences in DNA and RNA content was observed in some tissues, overall biodistribution remained consistent across groups. FIX expression was also consistent across groups. Anti-capsid IgG and IgM were assessed weekly, and while all groups reached high titers of anti-AAV IgG by the study termination, groups with higher percent of empty capsids consistently had accelerated IgG response compared to the 89% full groups for AAV8 and AAV9. Additionally, the typically transient IgM expression seemed to be prolonged in the AAV8 treated groups with more empty capsids. Microscopic evaluation of a representative organ spectrum was unremarkable for all groups.

This study demonstrated that biodistribution of systemically or CSF administered AAV is not impacted by changes in percent full capsid in mice. This indicates that percent full may not be of concern for efficacy, however impacts on the immune response to AAV capsid should be considered. The rapid onset of anti-capsid IgG, and potential prolonged IgM response, might increase the risk of immune complexes formation. This could lead to the activation of innate immunity and/or activation of the classical complement pathway. Further investigation is needed to assess the translational relevance potential impact on AAV administration safety and efficacy.

P0023

A Novel AAV Vector Design for Reducing Cross-Packaged ITR Promoter Activity

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Adeno-associated virus (AAV) vectors are viable tools for delivering therapeutic drugs to treat various diseases. Nevertheless, during their clinical development, it is essential to address impurities present in even highly purified AAV preparations. Impurities in the AAV packaging production include DNA, proteins, and empty capsids. Most of the DNA impurities, consist of viral plasmids and host cell genomes, can be digested by subsequent purification steps. But non-GOI DNA components encapsulated within the AAV cannot be efficiently eliminated. Non-GOI DNA contain bacterial elements in the plasmid backbone, involving antibiotic genes, bacterial replicons, and potentially open reading frame (ORF) sequences. These ITR guided mis-packaging contaminants are commonly referred to as cross-packaging. ITR is an essential component for viral packaging and replication and possess promoter activity. Adjacent plasmid backbone sequence can be driven for transcription and potential expression of unknown toxic proteins by ITR, which may both incite immune responses and bring safety risk for AAV products. The polyA signal is a DNA sequence that exerts a transcriptional termination effect and guides the polyA tail to attach to the post transcriptional mRNA. To mitigate the ITR-driven transcripts of cross-packaged AAV, we introduced polyA signal sequences (PolyA SS) proximate to ITR and developed a novel viral plasmid for AAV production. In addition, 3x overlapped stop codons (corresponding to +1/+2/+3 reading frames) was incorporated prior to the polyA SS to restrict possible translation from the unexpected transcripts, and these modifications are termed translation and transcription termination elements (TTTE). We evaluated the effects of TTTE on the transcription of the plasmid backbone *in vitro*. HEK293T were transduced with viral plasmids with the additional TTTE at upstream of the 5’ ITR or downstream of the 3’ ITR or both. Compared to parental plasmid, the insertion of TTTE at upstream of the 5’ ITR or downstream of the 3’ ITR reduced the backbone transcription products by 38% or 18%, and by about 87% when both insertions were applied. To confirm the TTTE effects *in vivo*, AAV were packaged through triple transfection system and administered into the tibialis anterior muscle of B6 mice. The modified and unmodified products were injected in the left and right legs respectively. Two weeks post injection, muscles were collected and analyzed for backbone expression. In vivo experiments demonstrated that TTTE modifications significantly reduced backbone transcription by more than 99%. These findings indicated that the introduction of TTTE into the upstream of 5’ITR and 3’ITR of AAV packaging plasmid can effectively inhibit the ITR-driven backbone transcription, which facilitates to increase the safety of AAV products.

P0024

ShuffleAnalyzer – a comprehensive tool to visualize DNA shuffling

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DNA shuffling is a powerful method for generating synthetic DNA via recombination of homologous parental sequences and has been widely used to create novel AAV capsids with altered properties. Shuffled DNA chimeras are often incorporated into complex libraries that undergo functionality screenings such as in vivo evolution, which ultimately allows identification of novel variants with improved characteristics in terms of tissue tropism or immune reactivity. To assess shuffling efficiency, subsequences of the chimeras can be computationally mapped to their parental origins, providing insight into the frequency of recombination events, the diversity of shuffling libraries and the actual composition of final variants. Although tools exist for parental assignment, they typically lack direct graphical visualization of the results making the analysis process time-consuming and cumbersome.

Here we introduce ShuffleAnalyzer, a comprehensive Python-based analysis tool that directly generates graphical outputs of parental assignments of shuffled protein or DNA sequences. ShuffleAnalyzer is a standalone tool with a self-explanatory graphical user interface that does not require any prior coding experience or a preinstalled Python version. It is provided as a single executable file for Windows, Mac and Linux and the entire code is available under a non-restrictive BSD-3 license. In addition to DNA shuffling, ShuffleAnalyzer can simultaneously analyze and visualize peptide insertions, making it a highly valuable tool for integrated approaches often used in AAV capsid engineering for gene therapeutic applications.

P0025

Biodistribution and immune response against CNS-targeting AAV vectors in non-human primates

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Adeno-associated viruses (AAV)-based gene transfer progressed in the last decade, up to the ability to treat single-gene disorders such as haemophilia A or spinal muscular atrophy with great successes. However, with the increase in clinical trials using AAV vectors, it now appears that AAV are more immunogenic than previously estimated, when used at high doses. Several clinical trials were put on hold after severe adverse events occurred, related to hepatotoxicity and upregulated immune response. To better bridge the gap between preclinical studies and clinical trials, we aimed to evaluate the biodistribution and immune response against three of the most-used AAV capsids, i.e. 6, 9 and rh10, to target the central nervous system (CNS) in non-human primates (macaca fascicularis). In order to test different routes of administration while minimizing the number of animals allocated to the study, the animals received combinations of vectors, each containing a fluorescent reporter. This experimental approach was first validated by intrastriatal injection into rats. All three vectors were detectable by qPCR and immunofluorescence when co-injected, allowing us to evaluate each vector tropism and biodistribution. Depending on the CNS areas to target, different administration routes can be used, such as intraparenchymal (IP) injection to target directly the injected area, or intrathecal (IT) injection for a more widespread targeting of the spinal cord. Six animals were included in the IP group and received a mix of the three AAVs in the left hemisphere (3E12 vg). To control for the effect of co-injection, a single AAV was injected in the right hemisphere (1E12 vg). Four animals were included in the IT group and received either a mix of the 3 AAVs or AAV9 only (3E13 vg). Blood and cerebrospinal fluid draws allowed us to monitor the clearance of AAV during the study and at day 28, animals were euthanized and tissues taken for analysis. When detecting the global viral load, IT delivery led to

higher viral load than IP and broader distribution in the peripheral tissues, notably the lymphatic organs (spleen, lymph nodes, dorsal root ganglia) as expected, but also in non-lymphatic organs (liver, kidneys and adrenal glands). For the IP group, the AAVs were detected in the injected brain areas as well as the spinal cord and dorsal root ganglia, but almost indetectable in the peripheral organs. The immune response data is now under analysis. This work will help to better characterize the influence of capsid type and injection route on vector biodistribution, and the immune pathways activated by AAV vectors, thus allowing the identification of targets for immunosuppression in clinical trials.

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P0026

Analytical development to measure DNA impurities in AAV gene therapy products

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Characterisation of critical quality attributes (CQAs) for recombinant adeno-associated virus (rAAV) gene therapies is required to assess quantity, purity, safety and potency of the therapeutic product. One of the big remaining challenges with this characterisation is the presence of DNA impurities introduced during the manufacturing process, both inside and outside of the AAV capsid containing the gene of interest. These impurities can have an impact on both the quality as well as the safety of a gene therapy product and have the potential to cause harm to a patient through immune responses, which can also reduce the efficacy of the therapy, or to cause carcinogenic affects. Currently the Food and Drug Administration (FDA) recommends a maximum of 10 ng/dose of residual DNA in gene therapy products.

Not only is the assessment of these impurities important for regulatory approval but also allows therapy developers insight into how their upstream and downstream processes are impacting the end products purity and raising areas for improvement. For the measurement of these impurities there are currently limited options available on the market and often associated with high cost. At Cell and Gene Therapy Catapult we have developed and qualified two PCR based assays to target both residual host cell DNA and residual plasmid backbone DNA as well as a Next-generation Sequencing (NGS) protocol to investigate DNA impurities in end stage rAAV drug products. The host cell DNA impurity assay was developed using a ddPCR platform, without the need for sample extraction and using the detection of human Alu sequences to determine the presence of host cell DNA. The residual plasmid backbone assay was developed using a qPCR platform targeting the kanamycin resistance cassette, with the potential for easy adaptation to target alternative antibiotic resistance cassettes. The NGS assay allows for the detection and characterisation of all possible DNA impurities in rAAV products giving information of their presence levels, their location, inside or outside the AAV capsids, and the size of the DNA impurities in base pairs. These three methods allow a comprehensive and sensitive analysis of the main contributors to DNA impurities in rAAV therapies, providing various avenues of information all important for product safety and quality.

P0027

Development of HPLC based methods for the systematic quantification of multiple quality attributes of AAV9 preparations

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There are many critical quality attributes (CQAs), such as aggregation, full/empty capsid ratio, viral protein ratio and other process-related impurities that need to be monitored to ensure consistent adeno-associated virus (AAV) product quality. However, due to their structural complexity, heterogeneity, potential instability and limited sample availability, characterization of AAV products remains challenging and typically requires a plethora of analytical techniques. Furthermore, the desire to quantify capsid titre and full/empty ratio in process during manufacturing remains somewhat unfulfilled as often these parameters are only calculated at the end of manufacture or rely on indirect measurements, such as capsid titre to vector genome ratio or A260/280.

In addressing some of these challenges, high-performance liquid chromatography (HPLC) based methods have become an integral part of the analytical toolbox. HPLC offers the reproducibility and throughput that other techniques struggle to reach, and when combined with multiple detectors (FLD and MALS) excellent sensitivity can be achieved. In particular, the application of MALS enables absolute estimation of aggregation characteristics (size, dispersity, particle counts, and molecular weights) with an improved sensitivity (signal is proportional to molar mass). Importantly, by measuring additional optical properties such as differential refractive index (RI) and light scattering (LS), it is possible to perform compositional analysis and orthogonally determine the full/empty ratio without the need for prior calibration.

In this work we developed an AEX-FLD method with a discontinuous gradient as well as a SEC-MALS method, both used for the quantification of empty and full capsids in AAV9 preparations. Both techniques have been shown to be precise, linear, robust, and accurate—correlating well with orthogonal methods such as analytical ultracentrifugation (AUC) and ddPCR. We demonstrated the possibility of applying both methods during manufacture for the determination of full/empty particles in in-process samples. Additionally, we verified the versatility of our approach by adapting the methods to quantify empty/full capsids in crude lysate samples.

P0028

Development of an *in vitro* potency assay for AVB-101, a recombinant AAV9 gene therapy for the treatment of frontotemporal dementia with progranulin mutations

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AviadoBio is developing AVB-101, a recombinant, AAV9-based investigational medicinal product for the treatment of frontotemporal dementia with progranulin mutations (FTD-GRN). The viral vector houses a therapeutic expression cassette containing a codon-optimised gene encoding human progranulin (*hPGRN*), driven by a neuronal specific promoter. Use of the latter element presents a major challenge in potency assay development, with negligible expression derived when using ubiquitous cell lines, such as HEK293 and HeLa. Whilst neuronal cell lines allow for transcriptional activity, many display low levels of promoter activity and inconsistent cell culture performance which makes them unsuitable as cell lines for use in routine QC testing. Furthermore, they show low permissiveness to AAV9, resulting in low levels of transgene expression which makes detection and quantitation highly challenging.

Here, we describe the systematic engineering of a HEK293 cell line using gene editing tools to enable AVB-101 potency assay development. Edits were designed to improve the permissiveness of the cell line to AAV9 and enable sensitive, quantifiable, and reproducible detection of hPGRN expression with no background.

In order to augment AAV9 transduction in HEK293 cells, first a gene encoding for a critical protein linked to AAV9 binding and cellular uptake was edited using CRISPR/Cas9-mediated inactivation. Several clones were isolated from the resulting pool and assessed for AAV9 transduction. Next, the endogenous *GRN* gene was disrupted in the clonal knockout cell line (HEK293$\triangle$*X1*) to abrogate expression of endogenous progranulin from the cell line. Once again, the modified pool was cloned, with the most promising candidate cells taken forward. Finally, the double-knockout, clonal cell line (HEK293$\triangle$*X1*$\triangle$*GRN*) was further modified to stably express a deactivated Cas9 (dCas9) fused to the transcriptional activators VP64, p65, and Rta (VPR), as well as guide RNA (gRNA) targeting the neuronal promoter sequence. Binding of the gRNA to the latter sequence elicits transcriptional activation, and thus *hPGRN* gene expression from AVB-101.

We have demonstrated a robust dose-dependent response in progranulin expression (by means of a progranulin ELISA), following transduction of these cells with AVB-101 at varying MOIs with high sensitivity and precision which are essential for potency assay development.

In summary, we have generated an engineered HEK293 cell line for the quantitative assessment of a key critical quality attribute of our gene therapy vector. Following further development and qualification, we expect this potency assay to supplant the current *in vivo* and qualitative assessments of progranulin expression.

P0029

Investigating the characteristics of AAV Rec2 *in vivo* and *in vitro*

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Adeno-associated viruses (AAVs) are immensely valuable tools in gene therapy. The possibility of engineering AAVs to alter their tropism and target specific cell types greatly contributes to their appeal in the field. One such engineered AAV, Rec2, has been shown to have improved transduction in adipose tissues *in vivo* when compared to existing AAVs. In this study, we aim to better understand the biodistribution of Rec2 *in vivo* as well as investigate its transduction

profile *in vitro*. Rec2 was packaged with a luciferase construct and administered *in vivo* using two different doses ($1x10^{11}$ and $1x10^{12}$ viral genomes/mouse), as well as application routes (intraperitoneal and intravenous) to determine their effects on its biodistribution. The resulting luciferase expression suggested that while the liver is transduced regardless of the administration route, the transduction of the adipose tissue seems dependent on it, with intraperitoneal delivery resulting in higher transduction when compared to intravenous delivery. To determine the transduction efficiency of Rec2 *in vitro*, a GFP construct was packaged into the virus and used to transduce human-derived adipose microtissues at multiplicities of infection (MOIs) $5x10^3$ and $5x10^4$. AAV2 and AAVDJ were used as positive controls, having a broad tropism and being efficient *in vitro* transducers. GFP expression for Rec2 was detectable only with the higher MOI of $5x10^4$ on Day 7 post transduction, while it was already detectable on Day 3 with AAV2 and AAVDJ. Taken together, our results highlight the importance of selecting the appropriate administration route depending on the application, along with the inconsistency of AAV transduction profiles *in vivo* vs *in vitro* as well as across species.

P0030

The possibility of novel adeno-associated virus (AAV) vector production system for gene therapy

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Adeno-associated virus (AAV) vector is a promising tool for gene therapy, and various clinical trials are ongoing in the world. However, there are many issues which should be improved for further development. Among various issues, tons of empty capsids during AAV vector production are one of the big problems, meaning that costly multistep purification is required to remove empty capsids. It leads to increased price for AAV vector-based drugs.

To improve this issue, we have established new AAV vector production system controlling capsid (Cap) expression timing after separating replicase (Rep)/ Cap gens in convention AAV vector production system. Our new AAV vector production system controlling Cap expression timing by the tetracycline-dependent promoter significantly increased the yield of AAV2 vector and improved the empty/full capsid ratio. In addition, our system could be applicable for various serotypes to improve the quantity and quality of viral vectors. Furthermore, our AAV production system could show the same improvement of vector productivity even in scaled up culture sizes such as T225 and Cell Factory multi-layer flasks. Moreover, the difference of AAV vector infectivity, produced by conventional and new system, could not be seen in various serotypes. There was also no difference of tissue tropism in mice using AAV9 vectors. These indicated that new AAV vector production system can generally improve AAV vector productivity in various serotypes without changing the nature of AAV vectors. Further analyses showed that the improvement of AAV vector productivity was due to additive effect of the change of Rep expression pattern by individual expression of Rep and the Cap expression timing control. Thus, our newly developed AAV production system has the potential to shed light on the reduction of cost for gene therapy.

In this presentation, we would like to share our findings, and discuss about the possibility of AAV production system and the further investigation of AAV biology in future.

P0031

Our modular, fully scalable AAV purification process enables vector recoveries of up to 50% with built-in vector safety

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The development of a fully scalable AAV purification process with high product yield and vector quality remains an important goal in the gene therapy field. Here we present the results of our modular and scalable AAV downstream process. The process was developed with AAV9 material produced with our proprietary suspension cell process. Scalable options for DSP development are mainly chromatography and filtration steps while the scaling of preparative ultracentrifugation is more complex. We have therefore focused on the former. However, these steps also have their limitations, as columns and filters are only available in certain sizes or dimensions. In order to create a suitable scale-down model for future process robustness studies, the column and filter sizes were increased for production scale. This may have an impact on product yield, but our data show that changes performed during DSP scale-up development did not negatively impact high product yield and quality. To increase product safety, several downstream steps to remove adventitious viruses were tested for AAV9 regarding product stability and recovery. These steps can be implemented in a modular way to meet future regulatory requirements without impacting product quality and quantity. Our 2-split plasmid system delivers AAV9 capsids with a %-full of up to 60 % (mass photometry) in upstream processing. We thereby implement high quality into the product from the beginning. A full-empty separation step, e.g. by AEX chromatography, might be dispensable for a number of applications which will be dependent on the intended dose, route of administration and overall risk/benefit profile of a given indication. Avoidance of full capsid enrichment results in higher DSP vector recovery. To further boost product quality, a high salt AEX chromatography in flow-through mode was developed as polishing step. By this step non-packaged process-derived impurities are further reduced, in many instances down to levels at or below the LOD/LOQ of our sensitive in-house analytical assays. To sum it up, we successfully developed a fully scalable DSP platform enabling vector recoveries of ≥50 % at high vector quality and built-in adventitious virus removal steps to meet future regulatory requirements.

P0032

Rapid and easy-to-automate AAV titer assays for cell line and bioprocess development

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In the past decade, the use of AAV (adeno-associated virus) as a vector in gene therapy has gained significant popularity, leading to an increase in projects and a pressing need for more efficient bioprocess development. To address this, PAIA Biotech has developed a rapid and robust capsid titer assay based on its proprietary microplate technology. The assay incorporates the proven *Thermo Scientific™ CaptureSelect™ AAVX* products and eliminates several steps from the

workflow of traditional microplate-based assays such as ELISA. This innovation allows for the measurement of hundreds of samples within one hour. It is particularly suited for applications in cell line development, enabling fast and reliable screening of AAV-producing cell clones. We present data for AAV serotypes 2, 5 and 8 and demonstrate the robustness of this assay against common matrix components such as cell culture media, salts and detergents.

P0033

AAV Cell Surface Target Profiling Using Binding and Functional Cell Microarray Screening Technology

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AAV (Adeno-Associated Viruses) vectors have emerged as a promising gene therapy. In 2019 > 200 clinical trials accounted for 24% of viral vector studies worldwide, however challenges remain in overcoming organ specificity, innate immunity, and dose-dependent toxicity. Here we describe a novel dual screening capability for AAV's, using the Charles River Retrogenix® Cell Microarray technology, to enable identification of novel cell surface receptor interactions involved in virus sequestration and functional payload delivery.

The Retrogenix® Cell Microarray consists of over 6,500 full length human cell surface, secreted and heterodimeric proteins, over-expressed in HEK293 cells. Now expanded to include non-human plasma membrane proteins with £ 90% human homology in the extracellular domain, the array can be used to identify novel primary receptors, potential off-targets and assess in vitro / in vivo efficacy and safety profiling for gene therapeutics. Additionally, a functional screening method has been developed enabling AAV binding to specific cell surface receptors and internalization (transduction) observed via reporter gene expression.
WT AAV9 controls presented over X cell surface interactions when tested, allowing distinction between sequestration and transduction activity upon cell surface binding. Examples include KIAA0319L, a protein receptor required for AAV endocytosis, together with AAV attachment factors and co-receptors, such as fibroblast growth factor receptors (FGFR), heparan based molecules, solute carrier proteins, interleukins and neuropeptides.

Functional and binding studies can facilitate candidate selection, demonstrate therapeutic specificity and de-risk future in vivo studies. This technology presents surface proteins in their natural environment, affording several advantages over conventional array technologies, including low false positive rates and high target specificity. AAV screening against Human and Non-Human arrays for potential off-target binding presents a unique strategy to help de-risk therapeutic candidates and provide supporting data for IND submissions.

P0034

Optimizing AAV peptide insertion library design for screening CNS tropic capsids

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Directed evolution approaches have been leveraged to engineer novel Adeno-Associated Virus (AAV) variants, specifically tailored for targeted gene therapy applications within the central nervous system (CNS). These studies necessitated the evaluation of a variety of peptide insertion library designs, with considerations given to aspects such as parental serotype, insertion location, and peptide length. In the current approach, we employed next-generation sequencing (NGS)-guided in vivo selection to assess the performance of three distinct AAV9 peptide insertion library designs: a 7-mer peptide, a 12-mer peptide, and a liver-de-targeted 7-mer peptide. Following each screening iteration, a thorough bioinformatics analysis was carried out to track the progression and to draw comparisons between CNS-derived peptides and those from off-target tissues.

NGS analysis of CNS-captured AAV variants revealed that the 12-mer insertion and liver-de-targeted 7- mer libraries exhibited improved performance compared to the unmodified 7-mer library. Subsequently, the CNS targeting performance of 28 selected mutant 7-mer and the 12-mer candidates were tested in C57BL/6 mice using both a barcoded AAV library approach and based on individual candidates. Among the 7-mer variants, less than 50% exhibited heightened CNS-expression relative to parental control vectors, while all the 12-mer candidates surpassed AAV9 in terms of CNS expression and liver de-targeting. The two 12-mer candidates, namely AAV9-CNS_005 and AAV9-CNS_006, showcased enhanced CNS transduction, outperforming AAV9 by ~26 and 40-fold respectively, and AAV9-PHP.eB by ~4-5-fold. Immunohistochemistry analysis corroborated that both AAV9-CNS_005 and AAV9-CNS_006, like AAV9- PHP.eB, primarily targeted astrocytes, endothelial cells, and neurons within the CNS, while minimal expression was observed with AAV9. Currently, we are in the process of analyzing single-cell transcriptomics method to determine the cell-type tropism of the novel CNS-tropic AAVs and assess their transducing capacities of the CNS in other mouse strains.

Besides the identification of novel potent transducers of the CNS in rodents, our study highlights several aspects of library design including the choice of peptide lengths and NGS-mediated monitoring of the screening process will help guide future screening attempts in higher species.

P0035

A stepwise approach to AAV full capsid enrichment

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The enrichment of full capsids within AAV (adeno-associated virus) bioprocessing is key to generating efficacious and safe AAV products. Empty capsids are linked to increased immunogenic responses, increased propensity to aggregation, and reduced transduction

efficiency necessitating higher dosing for clinical effect. Packaged and non-packaged capsids exhibit differences to their surface charges and in this research, we demonstrate how we have developed a toolbox to exploit this difference for empty/full separation on post-affinity purified material across different AEX (anion exchange) modalities. This approach has been demonstrated to work on multiple serotypes, utilising new commercially available products, and applying recent analytical tools to develop robust downstream bioprocesses.

DoE and OFAT techniques have been applied together with mass photometry to refine AEX empty/full AAV separation. Multiple AEX matrices were evaluated with different elution buffer mixes. Ultimately a process was developed which removed the requirement for elution gradients and have simplified the process to a 2-step elution method targeting the empty and full fractions specifically. This method has been transferred and implemented at both lab and GMP production scales, resulting in a separation between the empty and the full capsids and a 5-fold enrichment of the full fraction within our process.

P0036

Development of a triplex one-step RT-ddPCR method as a quantitative potency assay for the vMiX™ platform

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In vitro potency assays are essential in the development of any gene therapy product as they quantitatively measure the biological activity of the product. Potency is a critical quality attribute that must be part of the release and stability test panel for all medicinal products. A robust, reliable, and consistent potency assay is therefore essential to be able to test all manufactured batches and demonstrate the gene therapy vectors ability to deliver its genetic material and induce a therapeutic effect at the molecular level.

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disease that causes the loss of motor neurons, leading to progressive muscle atrophy, weakness, paralysis, and eventually death from respiratory failure typically within 3 years of symptom onset. Intermediate-length expansions in the Ataxin-2 (*ATXN2*) gene, are known to cause misfolding and aggregation of the Ataxin-2 protein in neurons, disrupting RNA processing and transcription, ultimately triggering neurodegeneration and significantly increasing the risk of developing ALS. Moreover, knockout and knockdown of ATXN2 in mice significantly slows disease progression in TDP-43 overexpressing mouse models, thus, silencing a gene like *ATXN2* has emerged as a therapeutic strategy to treat ALS.

AviadioBio has developed AAV9-miR-ATXN2 (AVB-205), a gene therapy product encoding a vMix™ gene silencing microRNA (miRNA) that targets and degrades *ATXN2* mRNA transcripts. The engineered miRNA is delivered via an adeno-associated virus serotype 9 (AAV9) vector. Once transcribed and processed, the miRNA binds complementary sequences on *ATXN2* mRNA, leading to its degradation or blocking its translation. By reducing ATXN2 protein levels, this approach aims to mitigate the toxic accumulation of proteins like TDP-43 to slow ALS progression.

Here we describe the development of a one-step RT-ddPCR *in vitro* potency assay, that can quantify the knockdown of endogenous *ATXN2* mRNA caused by the transduction of AVB-205.

For gene silencing approaches such as that employed by AVB-205, we demonstrate how measuring the knockdown of endogenous genes in relation to the multiple housekeeping genes in a single reaction could be used to assess product mechanism of action as well as providing a strategy for routine potency testing for batch release and stability.

The approach taken here can be applied across the vMiX™ gene silencing platform for rapid gene silencing potency assay development.

P0037

Enhancing AAV Productivity Through Helper Plasmid Design and Plasmid Ratio Optimization

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Harnessing rAAVs as viral vectors for therapeutic transgene delivery still requires improvements in yields and specificity to lower vector doses, and therefore manufacturing cost, as well as to improve patient safety. To this end, our research is focused on developing novel technologies to ensure manufacturing of high yielding rAAV particles using transient transfection, as well as enhancing viral particles quality and specificity. Here we present our state-of-the art approach to design new helper plasmids (pHelpers) with the aim of improving both the genomic titers and the infectivity (TU/mL) of the viral particle obtained from suspension cultures. We took the opportunity to exploit our proprietary DNA assembly method technology to explore the synergies of multiple genetic features modularly assembled in synthetic plasmids. We then explored how plasmid ratio optimization could further enhance AAV productivity through a Design of Experiment with a mixture design approach. This work led us to identifying plasmid ratios improving both VG titers and full / empty ratios.

P0038

Combinatorial targeting of adeno-associated virus (AAV) 2 vectors to fine-tune tropism

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Muscles and the central nervous system (CNS) are affected by numerous devastating and severe inherited diseases. However, due to their composition and size, or the difficulty of crossing the blood-brain barrier, they are challenging tissues for developing efficient and selective gene delivery tools. In response to this challenge, we are following a combinatorial approach by improving capsid variants identified by high-throughput screening of an AAV serotype 2 display library through rational design.

We screened our AAV2 peptide display library in 8-week-old C57BL/6 mice and isolated viral DNA from target (muscle and brain) and off-target tissues two weeks later. Individual sub-libraries were created from each target tissue. Prior to injection into mice for the second round of *in vivo* screening, libraries were spiked with capsid variants derived from previous screens. AAV variant-derived DNA and RNA were isolated from various organs and analysed by next generation sequencing. We thereby identified a set of six lead candidates showing minimal off-target effects in the liver, while exhibiting high specificity for CNS and/or muscle tissue. To test our initial hypothesis, lead candidates are currently being combined with additional rational design-based capsid modifications to enhance their targeting properties and transduction efficiencies. Assays will include human iPSC-derived cell models as well as large animal models. Moreover, we are conducting *in vitro* characterizations to assess capsid stability, pre- and post-entry mechanisms, and the ability to evade pre-existing anti-AAV neutralizing antibodies.

P0039

Generation and characterization of a HEK293 cell line for rAAV production

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Recombinant adeno-associated virus (rAAV) vectors are widely used in gene therapy applications. The most commonly used method for generating rAAV is the triple-transfection system in which three separate plasmids are transfected into mammalian or insect cells. Following a period of culture, rAAV vectors carrying the gene of interest are harvested for downstream applications. Both HEK293 (human) and Sf9 (insect) cells are the most widely used rAAV production platforms, although others have been described.

NewBiologix (NBX) has generated and characterized a new HEK293 cell line (NBX1-HEK293) specifically selected for the efficient transfection of plasmids and for rAAV production. Single clones derived from a polyclonal parental cell line were selected based on their high transfection efficiencies, growth profiles, capacity to grow as single-cell suspensions in selected culture media and ultimately rAAV production efficiencies following triple-plasmid transfection. The selected clones were then characterized both genomically using long-read sequencing (PacBio) and optical genomic mapping (Bionano), and transcriptomically using global RNA sequencing. A single clone was selected, and its performance was compared to that of commercially available HEK293cells that have been used in both pre-clinical and clinical development programs.

In conclusion, NewBiologix has generated and characterized a proprietary HEK293 cell line specifically optimized for the production of rAAV gene therapy candidates and which is available for licencing.

P0040

Development of a high sensitivity digital PCR assay for the in vivo quantification of transgene AAV DNA & RNA

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Recombinant adeno-associated virus (rAAV) is currently the most popular gene delivery vector for in vivo gene therapy. Part of the bioanalytical challenges in AAV-based gene therapy is related to the analysis of the biodistribution and pharmacokinetics of transgenic viral DNA and RNA. When screening candidate vectors or performing dosing studies of a drug, accurate knowledge about which tissues are infected and how efficiently is essential. We established a complete, semi-automated and highly sensitive digital PCR-based bioanalytical workflow for the in vivo quantification of transgene viral DNA and RNA starting from very small quantities of tissues. This workflow has been used for analyzing samples from several technology projects, and from both mice and NHP studies. The efficiency, sensitivity and accuracy of the quantification of viral genome in different tissues depends on the interconnection of several factors. Considerations related to the DNA/RNA extraction and purification conditions, effect of restriction digestion of the DNA template, digital PCR key parameters and housekeeping genes selection are presented.

P0041

Retargeting AAV2 and AAV9 to muscle cells by fusing an ARTC1-specific nanobody into the GH2/GH3 surface loop of VP1

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Nanobodies are single variable immunoglobulin domains derived from camelid heavy-chain antibodies. Due to their small size and high solubility, nanobodies can be readily fused to other proteins. Adeno-associated viral vectors (AAV) consist of an icosahedral capsid containing the transgene. The three capsid proteins VP1, VP2 and VP3 assemble the 60-mer capsid at a ratio of roughly 1:1:10. We previously showed that insertion of a membrane protein-specific nanobody into an exposed surface loop of VP1 can markedly enhance transduction of target cells expressing the corresponding membrane protein (1). We here report application of this technology to improve the transduction of muscle cells with AAV 2 and AAV9.

Despite recent advances in the field of AAV gene therapy, hurdles persist in utilizing AAV vectors for the treatment of severe genetic diseases such as Spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD) (2). Human as well as mouse skeletal and heart muscle cells overexpress the GPI-anchored ecto-enzyme ARTC1 (3). We genetically inserted an ARTC1-specific nanobody into the GH2/GH3 loop of VP1. AAV displaying the ARTC1-specific nanobody greatly enhanced the transduction of ARTC1-expressing cells *in vitro*. This paves the way for more effective AAV-mediated delivery of therapeutic proteins and/or RNAs to muscle cells in a wide range of diseases, and could thereby advance gene therapies.

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P0042

Imaging the rAAV manufacturing process: assessing the subcellular localisation of capsids during production

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Adeno-associated viruses (AAVs) are well-established gene delivery vectors. They are preferred over other viral vectors due to their excellent safety profile and their ability to efficiently transduce a wide variety of cell types. AAVs are typically produced by triple-transfection of mammalian cell lines such as HEK293 and although in recent years the manufacturing process has been subject to multiple improvements, production remains expensive and inefficient. Two of the major drawbacks of the conventional production systems are the high yield of empty capsids, which lack the therapeutic gene, and the low secretion of certain AAV serotypes capsids from producing cells.

While the cellular localisation of capsids throughout transduction and infection has been studied, not much is known about their distribution during the manufacturing process. In order to improve on the aforementioned drawbacks, an in-depth understanding of the AAV-producing cell environment is needed. This includes the identification of host-cell proteins which impact recombinant AAV (rAAV) packaging and egress, which can eventually lead to the development of engineered cell lines with improved production capabilities.

Here, we have designed a set-up to study the subcellular localisation of rAAV capsids during production in HEK293 cells using confocal microscopy and conventional immunofluorescence to identify cellular compartments of interest in which host-viral interactions might be important. Given the differences in rAAV production yield when using either adherent HEK293T or suspension 293F cells, we employed both cell lines for the study. We also assessed differences in localisation at different time points during production. As there are substantial differences in the secretion of certain serotypes, which could imply variability in their localisation during production, we decided to employ both AAV2, which is typically a non-secreted serotype and is mainly retained inside the cells, and AAV8 which is better secreted to the cell culture media. Since genome packaging is thought to occur into pre-assembled capsids, detection of intact capsids is fundamental. Therefore, antibodies that recognise only intact capsids of both serotypes were selected along with specific markers for a range of different organelles, to determine the subcellular localisation of AAV capsids. Moreover, to minimise the required volume of antibodies as well as to allow visualisation of multiple conditions on the same slide, we designed our experimental set-up in 8-well glass-bottom slides, which were pre-coated with 100 µg/ml poly-D-lysine to increase cell attachment of both suspension and adherent cell lines.

This set-up enabled the characterisation of cellular localisation of two AAV serotypes with different production characteristics in both suspension and adherent HEK293 cells in order to increase the understanding of the rAAV production cycle.

P0043

HEK293 suspension cell line development and optimisation for rAAV production

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The generation of cost-effective and reliable recombinant adeno-associated virus (rAAV) vectors is crucial for the development and success of AAV-based therapies in the advanced therapy medicinal product (ATMP) industry. Human embryonic kidney cell lines (HEK293) are commonly used for the production of rAAV particles and a variety of HEK293 cell lines are available from commercial suppliers. The Cell and Gene Therapy Catapult (CGTC) has developed its own HEK293 cell line for rAAV production, and as a tool for cell engineering, to support the development and manufacture of more affordable and effective rAAV therapies. The CGTC-HEK293 cell line was derived from an adherent HEK293 parental line that was subjected to suspension adaptation, clonal selection, expansion and screening for rAAV production, to then optimise a rAAV production platform around the final clone selected, which has enhanced its productivity.

P0044

In silico formulation screening of AAVs, a novel approach to early formulation development

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Drug Product (DP) formulation is critical in maintaining the (thermal) stability of adeno-associated virus (AAV) vectors during storage and administration. An optimal formulation buffer ensures that the viral particles remain intact and potent, preventing degradation and aggregation that could reduce therapeutic effectiveness.

There are several different AAV serotypes used for gene therapies due to their versatile tropism for infecting different cell types. AAVs show specific serotypes stability profiles, with e.g. differences in melting temperature for commonly used buffer types, as well as pH dependent thermal stability profiles that differ between the (wildtype) serotypes. The aim of this work is to use *in silico* formulation screening as a novel and efficient approach to early formulation development activities.

The AAV virus capsid consists of 60 viral protein (VP) monomers from VP1, VP2 and VP3, all derived from the same gene but using differential splicing variants and alternative start codons.

Due to its large molecular size, all-atom modelling of the entire intact AAV particles is computationally too demanding for most *in silico* modelling work (e.g., software packages). Course-grained molecular dynamics (CG MD) can be helpful in predicting the interactions between two full AAV virus capsids as an indication of aggregation propensity. CG MD however lacks the spatial resolution needed for *in silico* screening of the AAV virus capsids with e.g., the excipient(s) of interest. Alternatively, all-atom modelling on individual AAV capsid protein subunits is feasible, but such an approach loses part of its biological accuracy.

An alternative approach to molecular modelling of either (course-grained) full capsid or single VP capsids is to find a representative subunit of the full AAV virus capsid, which is computationally less demanding, whilst still resembling a biologically relevant presentation of the full capsid. Alternative approaches to molecular modelling of AAVs are to only extract amino acid residues that are exposed to the outer surface or by selecting a subset of VP monomers which form biologically relevant quaternary structures of the virus capsid. The cylindrical channel formed by a fivefold symmetric pentamer is such a promising AAV subunit as this channel has been reported to be required for endo/lysosomal capsid escape as well as ssDNA translocation.

Following the outlined approach, we aim to design and confirm (with wet lab experiments) a computational workflow that uses molecular modelling to screen excipients and physicochemical environment (e.g., pH) to find optimal formulations for AAVs. Once established, such a computational workflow allows for rapid screening of AAV for suitable buffer species, excipients, contact materials and surfaces providing valuable insights on the compatibility and stability profile of different (recombinant) AAV serotypes.

P0045

AAV-STARR-Seq screening platform yields cell type-specific enhancers and highlights the impact of core promoters and enhancer positioning

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Gene therapy using adeno-associated virus (AAV) has reached several relevant milestones in recent years. Yet, there is still a need for further improvements in terms of efficacy and safety. This can be achieved by implementing novel combinations of promoters and enhancers to increase specificity and expression strength of AAVs. For this purpose, functional high-throughput screening technologies, such as STARR-Seq (self-transcribing active regulatory region sequencing), have become pivotal tools. Here, we employed AAV-STARR-Seq to identify cell type-specific enhancers and scrutinized the impact of key aspects in experimental design on screening and validation outcomes. We constructed an *in-silico* candidate library focused on hepatocellular carcinoma (HCC) by integrating open chromatin and histone modification datasets. Candidate fragments were captured from sheared genomic DNA using biotinylated bait oligos and cloned into AAV-STARR-Seq expression cassettes. To assess the significance of core promoter choice for the screening outcome, we combined our library with three core promoters differing in strength and specificity. The screening libraries were applied side-by-side to two cell models of different tissue origin, HepG2 (HCC origin) and HaCaT (keratinocyte origin). Our screening results reveal that STARR-Seq activity was highly dependent on the core promoters used, and we

identified distinct sets of active candidate enhancers in HepG2 and HaCaT cells. Importantly, we could validate that our screening results yielded functional enhancer elements, which increased expression up to 60-fold in individual AAV-based luciferase reporter assays. Moreover, comparing reporter assays where the enhancer was placed either upstream of the promoter or downstream of the polyA site demonstrated that the level of enhancer activity is position dependent. These findings underscore the importance of considering both promoter dependency and position effects for screening and implementing enhancer elements into gene therapy approaches.

P0047

High-throughput screening of engineered ITRs to improve rAAV properties

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Gene therapy, a rapidly growing field of therapeutic research, aims to deliver genetic material to treat or prevent diseases. Among the various delivery vehicles, recombinant Adeno-associated viral vectors (rAAV) have emerged as one of the most promising due to their favorable safety profile, broad tropism, and stable episomal expression. However, AAV-mediated gene therapy faces significant challenges. Many systemically-delivered therapies require high doses of rAAV particles to achieve therapeutic efficacy, which can lead to clinical side effects. Additionally, even the most commonly used rAAV production platforms remain inefficient. Therefore, there is an urgent need to improve the quality of rAAV drug products, such as achieving high full-to-empty ratios, and to dramatically increase rAAV productivity and potency.

The element of the rAAV genome most closely linked to the packaging process is the inverted terminal repeat (ITR). Optimization of structural elements such as the B-arm, C-arm, and A-stem, as well as crucial functional elements for replication and encapsidation like the Rep-binding element (RBE), the terminal resolution site (trs), and the D-sequence, can significantly improve manufacturing yield as well as influence the overall efficacy of AAV-based therapies.

This study aims to evaluate various ITR variants and identify modifications that yield optimal performance. To systematically assess these ITR variants, we established a high-throughput screening platform capable of producing and evaluating rAAV particles with different ITR configurations and modifications in small scale HEK293 suspension format.

Initially, we designed and tested 9 distinct ITR variants, each with specific alterations in the B-, C-arm and A-stem composition, CpG content, and serotype compatibility. These variants were assessed in three configurations: (1) both 5' and 3' ITR regions modified in parallel, (2) only the 5' ITR modified, and (3) only the 3' ITR modified while deleting the second ITR entirely. Analytical assessment involved dPCR quantification for viral genome titer determination, next-generation sequencing in order to verify rAAV genome integrity as well as functional screening.

The first phase of screening revealed certain structural formations within the ITRs that significantly impact rAAV packaging efficiency and transgene expression. Variants with one ITR entirely deleted on either side of the genome exhibited dramatically reduced viral genome titers and functionality. Notably, certain full-length wild-type ITRs (145 bp) in configurations (2) or (3) demonstrated superior packaging efficiency compared to shortened variants. However, the performance was restored by the addition of a second ITR variant. Leveraging these insights, we designed a second batch of ITR variants focused on functional optimization rather than strict

adherence to structural elements. This led to the creation of an interchangeable ITR toolbox, allowing for flexible and context-specific optimization of rAAV vectors.

This study underscores the potential of ITR engineering to improve rAAV packaging efficiency and functional efficacy, paving the way for more effective gene therapies. Our high-throughput screening platform successfully identified key structural and functional elements within ITRs. The development of an interchangeable ITR toolbox represents an advancement in the field of AAV gene therapy, providing a versatile and robust approach for optimizing vector performance.

P0048

Understanding recombinant AAV production kinetics in HEK293 cells

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Recombinant AAVs are important gene delivery vectors due to their safety profile, low immunogenicity and tissue tropism among other relevant features. Because of this, the number of AAV gene therapy development pipelines have considerably increased in industry and academic groups worldwide since the approval of first AAV-based gene therapies (Glybera approved by EMA in 2012, Luxturna approved by FDA, 2017). Moreover, the gene therapy market is expected to experience a remarkable increase in demand the coming decade. Despite the predicted increase in global demand, AAV-gene therapy manufacturing is far from being efficient. One of the major contributors to the low efficiency of the manufacturing process is poor viral genome replication, packaging and egress, leading to high number of empty capsids or truncated-DNA filled capsids that need to be removed during downstream processing. This inefficiencies in the manufacturing process, dramatically increase the production costs, placing AAV-gene therapies among the most expensive pharmaceuticals and therefore, compromising accessibility to the patients in need. In order to address this problem, a better understanding of AAV production biology and kinetics is needed. Here we present a preliminary study in which we analyse HEK293 specific productivity together with Rep protein expression for recombinant AAV2 production at different timepoints after triple transfection. This study will set up a framework for future analyses of the effect of different elements of AAV production process in the host cells, opening new possibilities for cell line engineering to improve AAV production.
Moreover, we reflect on the need of reliable analysis methods for quick characterization of recombinant-AAV vectors in early stages of research and development, and we present technical challenges that need to be considered when quantifying viral genome titers in crude cell lysates using qPCR.

P0049

Advancing Directed Evolution: Machine Learning-Guided Optimization of AAV Variants Screening

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Adeno-associated virus (AAV)-mediated gene therapy has emerged as one of the most promising platforms for gene therapy. However, naturally occurring AAVs often exhibit off-target issues, leading to the use of recombinant AAVs to enhance tropism and reduce off-target effects. Many researchers employ AAV libraries and directed evolution for variant screening, but these methods may not encompass all possible sequence areas, potentially missing sequences with most promising tropism. To address this issue, we integrated directed evolution with a machine learning-based strategy to identify AAV variants with enhanced tissue tropism. In this study, we constructed randomized libraries to determine their tissue-specific tropism. After in vivo selection in mice, we utilized these AAV libraries in an Illumina sequencing-based next-generation sequencing (NGS) approach. We then employed machine learning techniques, including visualization algorithms, to discover crucial amino acid properties that enhance tropism to specific tissues. Furthermore, we identified global optimum values using explainable AI (XAI). By leveraging this machine learning-based approach, we can identify previously unreachable sequences, highlighting the significant role of machine learning in advancing gene therapy.

P0050

Continuous AAV mass production -One step producer cell line establishment with originally designed novel plasmid-

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Recombinant adeno-associated virus (rAAV) vectors are popular emerging modalities for gene therapy. Current manufacturing processes using plasmid-polymer complex transfection are expected to be insufficient to meet the demand for the estimated patient numbers, due to difficulties in controlling the plasmid-polymer complex during manufacturing . The high cost of the plasmid itself is another limiting factor. To solve these problems, we have developed "AAV stable producer cell lines" in which the genes necessary for AAV production were incorporated into the chromosomes of the production cells and established a method for high production of AAV vectors using these cell lines.

A unique design was constructed in which the genes required for AAV production, REP/CAP/Helper/GOI, and a drug-inducible switch was incorporated to avoid cellular damage caused by AAV gene expression were carried in a single plasmid. The Plasmid was integrated into the HEK293 cell chromosomes, and stable cell lines were established within 2 months. The AAV producer cell lines showed good proliferation comparable to the parental HEK293 cells without

inducers. The culture was expanded in the bioreactor and reached high cell density, and then >1E12 vg/ml AAV vectors were produced by adding the inducer.

Furthermore, the cells were withdrawn from the perfusion reactor every 24 hours for 7 consecutive days, and high productivity were confirmed for all batches on all days. With the new cell construction method shortened by using One Plasmid, this technology could contribute to Vector production at various scales.

P0051

Characterization of a safe and functional GT-GJB2 vector for the treatment of DFNB1A hearing loss

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The estimated prevalence of severe to profound deafness is 1 in 1000 neonates worldwide, with genetic factors accounting for half of the cases. Pathogenic variants of GJB2, the gene encoding for Connexin 26 (Cx26 also known as Gap Junction protein Beta 2), are involved in 50% of congenital deafness and are mostly associated with an autosomal recessive non-syndromic form, DFNB1A. No specific treatment exists for these patients besides cochlear implantation, which provides restorative benefits but does not fully replicate the clarity of normal hearing. Gene therapy is a promising therapeutic avenue as it aims for long lasting restoration of natural hearing function.

Connexin 26 hexamers oligomerize into hemichannels or connexons that connect head-to-head to form an axial channel or gap junction between adjacent cells, thereby allowing the interchange of small molecules such as nutrients, ions, and signaling molecules. GJB2 plays an important role in the recycling of potassium ions, which is critical for the maintenance of endocochlear potential and cochlear functions. In the cochlea, GJB2 is largely expressed in the supporting cells (SCs) of the inner and outer sulcus as well as in fibrocytes and lateral wall regions associated with the stria vascularis. Importantly, sensory hair cells (HCs) are devoid of connexin 26, and the ectopic expression of this protein has been reported to be toxic. A major challenge for successful GJB2 gene therapy is therefore the development of a tool allowing precise expression of the transgene in the intended cells with very limited off-target. Here, we combined an AAV with strong tropism towards a broad range of cochlear cells and a cassette that includes proprietary cis-regulatory elements to prevent transgene expression in sensory hair cells. *In vitro*, our therapeutic vector, hereby referred to as GT-GJB2, efficiently transduces human cell lines to produce Cx26 protein that is adequately addressed to the plasma membrane. Using an adapted dye transfer assay, we show that the transgenic Cx26 protein is functional and permits the passage of the fluorescent tracer to neighboring cells. Intracochlear injection in mice and NHP results in transgene expression in most GJB2-expressing cells along the tonotopic axis, with good local and systemic tolerability. In all analyzed cochleae, no sensory hair cells expresses the transgene, confirming the specificity of our cassette. Long term safety studies conducted in wild type mice demonstrate prolonged persistence of transgene expression with no impact on auditory function and maintenance of the cochlear cytoarchitecture. Additionally, intravenous injection of GT-GJB2 in mice does not induce any adverse events or changes in behavior, vital functions, increase in hepatic damage or inflammatory biomarkers in the tested animals.

In conclusion, we have developed a specific AAV vector/expression cassette combination that leads to strong and durable expression of Cx26 in cells of interest in the cochlea, with virtually no off-target detected. The vector is safe over an extended time after intracochlear administration in mouse or NHP models and represents therefore an excellent candidate for Sensorion's GJB2 gene therapy program.

P0052

Preclinical development of GT-GJB2 as a treatment for the autosomal recessive non-syndromic deafness 1A (DFNB1A) using an adeno associated vector-based gene therapy

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In the world, the estimated prevalence of severe or profound deafness in human is 1 out of 1000 neonates, and genetic factors account for half of the cases. Pathogenic variants of GJB2, the gene encoding for Connexin 26, are involved in 50% of congenital deafness and are mostly associated with an autosomal recessive non-syndromic DFNB1A. In the cochlea, GJB2 is largely expressed in the supporting cells (SCs) of the sensory epithelium, fibrocytes, and basal and intermediate cells of stria vascularis but not in sensory hair cells. It is hypothesized that Cx26 is essential for the recycling of potassium, which is essential for the proper functioning of sensory hair cells, but in vivo studies also suggest that Cx26 deficiency leads to cochlear developmental disorders. Gene therapy is a promising therapeutic strategy for autosomal recessive forms of deafness, and Adeno-Associated Vectors (AAVs) are being developed to this aim. Here, we have developed GT-GJB2, an Adeno-Associated Virus (AAV) vector for DNFB1A that offers broad coverage of *Gjb2*-expressing cells of the inner ear in both mouse and non-human primates. GT-GJB2 was delivered into congenitally deaf conditional *Gjb2* mutant mouse ears through the round window (RW). Intracochlear injections of GT-GJB2 into conditional *Gjb2*-mutant mouse inner ears lead to improvement of hearing thresholds as early as 3 weeks post-injection in a dose dependent manner. Efficacy on on-going cohorts, dose-response experiments, early biodistribution and toxicology studies in mice are under investigation. In parallel, GT-GJB2 was administered to Non-Human Primates (NHP) using the surgery and device that will be used in human. Early tolerability and biodistribution of GT-GJB2 studies were conducted in both species. Three weeks post-surgery, ABR measurements and DPOAE amplitudes remained contained within the normal hearing threshold range of NHPs indicating that GT-GJB2 was well tolerated. Whole mounts and cryosections of injected inner ears were analyzed to assess the AAV tropism. For both products, a vast majority of SCs that naturally express *GJB2*, including the great epithelial ridge cells, the lateral epithelial ridge cells, border cells, phalangeal cells, pillar cells, fibrocytes of the lateral wall and spiral limbus were transduced along the tonotopic axis. No transduction was found in inner hair cells. GT-GJB2 allows to efficiently and safely target the cells that naturally express GJB2 in the cochlea with both tropism and levels compatible with therapeutic intervention in human. These data support GT-GJB2 development and constitutes a major step toward our future clinical trials to restore physiological hearing in DFNB1A patients.

P0053

A Highly efficient PCR-based Approach to isolate Novel Naturally-Occurring AAVs

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Adeno-associated virus (AAV) vectors, renowned for their exceptional efficacy and safety, stand as the leading platform for in vivo therapy. To uncover new AAV variants with distinctive biological features, PCR-based amplification of AAV capsid sequences from biological samples has been mostly focused on short variable regions of the Cap gene (here called DgPCR for (diagnostic PCR).Here we describe an efficient PCR-based method that amplifies in a single step the entire Cap coding sequence (here called Full PCR) thus allowing to identify novel variants with differences across the whole capsid which we used to screen a collection of porcine liver samples. Using the traditional Dg PCR which targets three hypervariable regions (VRs) in the CAP gene, 36% (n=98/273) of liver samples were found to contain AAV sequences. Amino acid sequence alignment of these fragments revealed 7 novel AAV variants among the positive AAV samples. When the liver samples were analyzed with Full-PCR, 25% (n=69/273) of all samples were positive for AAV presence, however 17 of them were novel variants (including the 7 identified by DgPCR) based on aminoacid differences across the whole CAP and not limited to the hypervariable regions. All 17 variant CAP sequences were vectorized resulting in high titer AAV vector preps. We conclude that Full PCR is more effective than DgPCR for the identification of novel AAV variants from biological samples allowing to isolate in a single step the whole CAP sequence for further rapid vectorization.

P0054

A new perspective on AAV-mediated *in vivo* direct reprogramming: incorporating exogenous variables beyond cellular interaction

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In vivo direct reprogramming has emerged as a promising approach for regenerating damaged tissues by converting resident cells, thus obviating the necessity for invasive cell transplantation. Successful therapeutic outcomes with this technology requires efficient gene delivery vectors. Prior research on gene vectors has predominantly focused on identifying vectors that can selectively and effectively target specific cell types. However, in practice, various factors influence the efficacy of viral gene delivery, including interactions between cell receptors and vectors, as well as the complex in vivo environment that the vector must navigate to reach target

cells. This study hypothesizes that upon in vivo administration, each virus exhibits distinct behavior in response to diverse environmental variables at spatial scales exceeding the cellular level. To test this hypothesis, we conducted in vitro experiments using ECM-like structures to analyze various types of adeno-associated viruses (AAVs). Subsequent in vivo studies utilized ECM-like scaffolds to enhance therapeutic efficacy. Our findings indicate that a comprehensive understanding of how the target tissue region and its specific characteristics influence AAV delivery can enhance its efficacy. Furthermore, leveraging this understanding to modulate viral characteristics could potentially improve therapeutic outcomes. This research underscores the importance of considering the broader environmental context in gene therapy and suggests that strategic adjustments of vector properties can enhance the precision and efficacy of in vivo direct reprogramming.

P0055

High-Throughput Screening Platform Using Retinal Organoids to Optimize AAVs for Gene Therapy

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Recombinant adeno-associated virus (rAAV) vectors are commonly used for gene therapy, especially in treating retinal diseases. Optimizing AAV vectors for a specific gene therapy application involves careful consideration of various factors, including the selection of a promoter sequence that drives expression at appropriate levels exclusively in the desired cell types. Due to current gaps in understanding the precise regulation of cell type-specific expression, many promoter variants must be tested to identify the ideal one for treatment. Traditional methods of testing AAV variants on human retina explants from post-mortem donors or animal models are limited by availability and labor-intensive processes, restricting large-scale testing.

To address these limitations, we developed a high-throughput screening platform using human retinal organoids that mimic the human retina's diverse cell types. This platform allows parallel evaluation of numerous AAV variants, enhancing efficiency and scalability. The process involves cloning AAV plasmids with different promoter variants that drive GFP expression in a 96-well plate format. We then produce AAV vectors in high-throughput, generating sufficient quantities to transduce retinal organoids. Post-transduction, live imaging measures GFP expression in the organoids, and promising candidates are selected for detailed analysis in organoid sections using cell type-specific markers.

Our platform demonstrates versatility in identifying new promoter sequences for targeting specific retinal cell types, with potential applications extending to other vector properties and organoid model systems.

P0056

UpTempoSM Plasmid Platform Process Featuring a Single Chromatographic Step

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Plasmid DNA (pDNA) serves as a critical starting material to produce advanced therapy medicinal products (ATMPs) in the fields of cell and gene therapy. The growing demand for pDNA requires the development of manufacturing processes that can meet aggressive timelines while maintaining cost-effectiveness. Here we present the UpTempoSM Plasmid Platform Process, a robust single-use process that enables seamless scale-up from research and development (R&D) to good manufacturing practice (GMP) grade. A key feature of this platform is a single chromatographic step (purification and polishing) enabled by a controlled lysis that results in process simplification and cost reduction. Thanks to this process, Catalent is able to provide high quality and quantity pDNA for different types of cell and gene therapies.

We will provide an overview of the platform process, share results on the quality attributes obtained through its implementation and how this platform integrates into Catalent UpTempoSM AAV platform timelines. Our findings demonstrate the effectiveness of this approach in generating pDNA that meets quality requirements set by regulatory agencies.

P0057

Successful validation of capsid titer and host-cell derived DNA impurity assays extends our rAAV batch release QC portfolio

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Quantification of the capsid titer and packaged host-cell derived (HCD) impurities are critical quality attributes for rAAV batch release.

AAV9 capsid titer determination was established on the automated immunoassay system Gyrolab xPlore. Commercially available AAV9 empty capsids from two different manufacturers were used as standard and trending control, respectively. In robustness studies, usage of different lots of empty capsids for standard and trending control, and kit components was addressed.

For HCD size determination, we established a droplet digital PCR (ddPCR) targeting the 18S ribosomal RNA gene locus serving as a surrogate gene for packaged DNA impurities in rAAV. Before analytical validation, the method was qualified and tested for several parameters like droplet lifetime and sample dilution storage in intensive robustness testing. Robustness studies of

both methods were carried out in accordance with ICH Guideline Q14 – Analytical Procedure Development.

In our GMP laboratories, analytical validation of both methods was performed in according to ICH Guideline Q2(R2) – Validation of Analytical Methods. Here we present the results of robustness testing and analytical validation addressing specificity, working range including suitability of calibration model and lower range limit verification, precision, and accuracy of the methods. Since both platforms include the possibility of analyzing different serotypes (capsid titer) and rAAV batches from different production cell lines (HCD impurities) with minor adaptions of the protocols, it offers high potential to extend our portfolio for customer rAAV batch release testing.

P0058

Utilising High Throughput Process Development (HTPD) and Design of Experiment (DoE) to Optimise the Pharmaron Gene Therapy Platform

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At Pharmaron, we have successfully developed a platform process for the creation of AAV gene therapies with a highly adaptive toolbox to manage varying AAV products and serotypes. Our toolbox can rapidly assess a product's fit to our platform and finely tune a number of parameters for targeted optimisation. Pharmaron's use of HTPD and DoE allows fast screening and robust optimisation of our process to suit specific gene therapy product needs, leading to reduced time to clinic.

P0059

Nanopore sequencing grants detailed insights into a small molecule's impact on encapsidated DNA composition during manufacturing platform development

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Adeno-associated viruses (AAVs) have emerged as promising vectors for gene therapy due to their low immunogenicity, ability to transduce a wide range of tissues, and persistence of gene expression. As their clinical success has increased the global vector demand, enhancing AAV manufacturing efficiency while maintaining or further improving vector quality and potency is critical for advancing gene therapy applications. We have previously shown that the addition of small molecule SM-016 led to significant improvements in AAV9 vector genome (vg) yield in multiple production systems (Derler et al., Poster #328, ESGCT 2023). Here, we investigated the

effect of SM-016 on vector integrity and impurity profiles of an rAAV9-SEAP vector batch manufactured in an AMBR15® bioreactor using our proprietary long-read nanopore sequencing platform.

In the rAAV-9-SEAP reference, more than 96% of the total reads generated by nanopore sequencing mapped to the vector genome, with a significant proportion of full-length reads spanning from ITR to ITR. The addition of SM-016 did not impact the vector length distribution, but it resulted in a slightly increased level of plasmid-derived impurities. Closer inspection of the profiles revealed an increased impurity packaging by ITR readthrough or reverse packaging, whereas p5 promoter reverse packaging was less affected. The addition of SM-016 did neither impact host cell DNA (HCD) impurity amount, nor its length or chromosome distribution.

As SM-016 increased encapsidated DNA impurities, we tested if the application in two halved doses instead of a single full dose can alleviate the impact on quality. However, when the small molecule was introduced in a split dose regimen, only a marginal reduction of plasmid backbone impurities compared to the single dose was noted.

In summary, we demonstrate an effective approach using long-read nanopore sequencing, that provides profound insights into rAAV batch characteristics and especially DNA impurities. We showcase how process development can be guided at the example of SM-016 that enhances rAAV9-SEAP yield. Nanopore sequencing revealed no impact of SM-016 on vector length distribution, helper plasmid and HCD impurities. A slight increase in plasmid derived impurities was detected with data generated guiding on the development of mitigation strategies.

P0060

Amyotrophic Lateral Sclerosis (ALS): Unveiling the role of oligodendrocytes in disease progression and therapeutic strategies

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ALS is a relentlessly progressive neurodegenerative disorder. It attacks motor neurons, the nerve cells responsible for controlling muscle movement leading to loss of muscle function, paralysis and death. While the exact mechanisms of ALS pathology remain to be fully understood, recent research is shedding light on the intricate relationships between cellular compartments within the nervous system that can potentially contribute to the disease.

In fact, while traditionally, the focus has been on the loss of motor neuron function as the primary driver of ALS progression, exciting new discoveries are revealing pathological deterioration of the skeletal muscle cell in the periphery and also astrocytes and microglia cell populations resident in the spinal cord. Our research using single-cell RNA sequencing analysis of the spinal cord in SOD1-G93A mice, a well-established model of ALS, revealed a significant decline in the oligodendrocytes, responsible for generating the myelin sheath, a fatty insulation that protects and speeds up nerve impulses along axons. This finding was further validated by immunostaining for myelin basic protein (MBP), an oligodendrocyte marker, in SOD1 mice, confirming a decrease in these supportive cells.

To counteract this loss, we have developed a viral vector (AAV-10) carrying an oligodendrocyte-specific promoter, and the transcription factor SOX-10 needed for oligodendrocytes

differentiation. Our previous work using AAV10-U7-SOD1 gene therapy in the SOD1-G93A transgenic mouse model showed promise in eliminating toxic SOD1 accumulation. However, oligodendrocytes are not efficiently targeted and warrant a different approach. Our preliminary results show an efficient targeting of this cell population after intra-central nervous system injection of the viral construct and an investigation is ongoing to evaluate the therapeutic efficacy of this approach alone and in combination with the previously tested gene therapy.

By addressing oligodendrocyte dysfunction and promoting their maturation, we aim to develop a novel therapeutic strategy aiming not only to target motor neurons but also to enhance axonal integrity, potentially delaying disease progression and improving the quality of life for ALS patients.

P0061

High Sensitivity Capillary Electrophoresis Analytics for End-to-End Plasmid and AAV Purity Determination

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Pharmaron Biologics (UK) is a fully integrated cell and gene therapy development and manufacturing organisation specialising in the end-to-end production of plasmid DNA and recombinant adeno-associated virus (AAV) vectors. The state-of-the-art facility enables the extensive analysis of plasmid DNA and AAV critical quality attributes (CQAs) for the production and release of gene therapy products.

Plasmids, used as a critical starting material in the manufacturing of AAV, require a high degree of supercoiling for maximum transfection efficiency and AAV yields. Furthermore, AAV purity is a CQA to demonstrate optimal viral protein expression and expected viral protein ratio. It is therefore imperative to monitor plasmid isoform distribution and AAV viral protein expression to determine the purity. Capillary Electrophoresis coupled with Laser Induced Fluorescence (CE-LIF) provides a rapid, sensitive, and reproducible method for the purity analysis of plasmid DNA isoforms and AAV viral protein. The current CE-LIF platform methodologies available allow for the purity analysis of in-process, purified plasmid and multi-serotype AAV drug substance, in various sample matrices. This technique offers reduced sample requirements, improved accuracy, and superior resolution in comparison to more traditional analytical purity methods, such as agarose gel electrophoresis. Moreover, the recently launched SCIEX BioPhase 8800 multi-capillary CE system has enhanced Pharmaron's analytical capabilities, with 800% increased sample throughput and considerable reductions in method development time.

Here, Pharmaron present an overview of their successfully validated low volume platform CE-LIF methods which are readily available for the purity determination of a range of plasmid sizes and AAV serotypes throughout the production process. All requiring minimal optimisation for fast delivery of high-quality gene therapy products to patients.

P0062

Engineered AAV9 variants for kidney targeting

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Adeno-associated virus (AAV) vectors have been approved for treatments of many genetic disorders, and are widely used in clinical gene therapy trials. However, targeting genetic kidney diseases, which account for approximately 30% of patients with chronic kidney disease (CKD), remains challenging, mainly due to the lack of renal cell-specific AAV. We therefore generated an AAV9 peptide display library and performed viral vector selection in the kidney using a nephrotic mouse model. A galactose binding-deficient AAV9 library was used to avoid liver targeting, while the nephrotic model with a compromised glomerular filtration barrier should facilitate the AAV library's access to various glomerular cells, such as podocytes. As a result, after three rounds of selection in mice upon intravenous administration, we identified a new variant with an enhanced targeting the juxtaglomerular apparatus in the kidney, while showing very low liver targeting. Moreover, to target other kidney cells, such as glomerular endothelial cells, the incorporation of the AAV9 galactose-binding domain is essential. In summary, we successfully identified kidney-specific AAVs by evolutionary selection in mice and AAV9 galactose-binding domain modification, enabling kidney gene therapy in the future.

P0063

Development of anion-exchange chromatography (AEX) process by comprehensive analysis for AEX-eluted fractions in adeno-associated virus vector manufacturing

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Adeno-associated virus (AAV) is very promising as a viral vector and is at the forefront of gene therapy and regenerative medicine. Through the manufacturing process, however, not only full particles, which encapsulate whole length of the target gene (GOI), but also empty particles, which do not encapsulate the GOI are produced at the same time. The problems are the possibility to diminish the therapeutic efficacy and to enhance immune responses. Purification methods using anion exchange chromatography (AEX) have attracted attention as a safe process to remove empty particles for clinical applications, and various approaches have been taken to improve the ratio of full particles to the total AAV particles (F/E ratio). In this study, each AEX-elution fraction was analyzed to efficiently search for conditions for full particles separation by AEX. The following analytical methods were used. SDS-PAGE was used for protein impurity analysis, Capillary electrophoresis with laser-induced fluorescence (CE-LIF) for nucleic acid impurity analysis, Dynamic light scattering (DLS) for size distribution, Electrophoretic light scattering (ELS) for zeta potential, and Mass photometry (MP) for F/E ratio analysis. The combination of these analyses revealed the composition and state of particles in each elution fraction. Based on this information, we are aiming to improve the F/E ratio by AEX purification.

P0064

Applying next generation sequencing (NGS) to accelerate cell and gene therapy product development

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Viral vector mediated gene therapy (GT) vectors, like AAV, are typically aimed at conditions for which no other treatment exists and for which there's urgent need. Viral vectors are also critical for the manufacture of many cell therapy products. Here, we outline where NGS can be applied during development and manufacture of cell and gene therapies to accelerate development and gain greater insights into product characteristics to de-risk the development process.

Cell banks used to manufacture viral vectors require extensive viral safety testing including broad spectrum virus detection assays. NGS is now recommended in key global regulatory guidelines such as ICH Q5A (R2) as an alternative to traditional *in vivo* assays for adventitious virus detection. We present how fully validated GMP compliant NGS assays incorporating custom data analysis algorithms for virus sequence detection are used to provide viral safety assurance for cell banks required to manufacture AAV and viral vectors needed for cell therapy manufacture, such as Lentivirus.

From early stages of GT manufacturing process development, testing to unequivocally confirm genomic identity is a regulatory requirement, with both the FDA and EMA recommending determining the entire vector sequence. We demonstrate how NGS enhances the depth of insight into potential sequence variants (insertions, deletion and substitutions) and their relative frequency, providing risk mitigation as well as accelerating the process by removal of the need for sequence specific primer design. NGS can also provide valuable insights into viral vector key quality attributes that may not be easily achieved with other techniques, such as characterisation of non-target encapsidated nucleic acid in AAV particles. We present data obtained for multiple AAV serotypes, demonstrating how product-related impurities such as residual host cell DNA and plasmid DNA can be detected using NGS (both short-read and long-read technology), with the ability to determine composition and relative proportions of nucleic acid impurities as compared with the intended target packaged DNA.

Lentiviral vector-mediated therapies exploit the ability to integrate into non-dividing cells to deliver and stably integrate transgenes to create cell therapies such as CAR-T. In addition to the applications for safety testing of MCBs and vector identity testing, NGS can also be used to determine safety attributes of final cell therapies, such as lentiviral integration site analysis, which is critical to investigate potential genotoxicity following random integration events. Recent FDA guidance on Gene Therapy Products incorporating Gene Editing (2024) outlines expectations for determining both on- and off-target editing activity, for which NGS will be a critical component of the toolbox for safety testing of edited cells. Similarly, recent *draft* FDA guidance on Safety Testing of Human Allogeneic Cells (2024) recommends whole genome sequencing of cell populations contributing to the final product as well as the edited cells themselves, which will necessitate NGS to evaluate potential mutations, genomic integrity as well as integration sites and on-/off-targeting events. Data presented will outline examples of the expanding role of NGS in advancing development of cell and gene therapies

P0065

Optimizing AAV Vectors for Precision Gene Therapy: A Rational Design Approach

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Genomic medicines represent groundbreaking therapeutic approaches for treating diseases with limited therapeutic options by delivering genetic material directly to patient cells. Various vectors serve as delivery vehicles, including viral and non-viral vectors such as lipid nanoparticles (LNPs). Adeno-associated viruses (AAV) are particularly favored in genomic medicines due to their extensive tissue biodistribution, which allows them to target a broad range of diseases. However, this broad tropism can result in a lack of tissue specificity. Therefore, optimizing capsid structure is crucial for enhancing AAV specificity, selectivity, manufacturing efficiency, and reducing immunogenicity. Current AAV capsid engineering efforts aim to improve the vector delivery efficiency to target cells.

A common way to improve AAV capsid engineering is to design vectors that enhance their specificity for target receptors. This approach leverages AAV vectors' inherent ability to tolerate mutations and peptides insertions in specific viral protein regions, notably the variable regions (e.g. VR-VIII). Despite significant advancements in protein structure prediction methods, in-silico peptide biologics design remains challenging. However, peptide design is promising for enhancing the targeting capabilities of AAV vectors. To address this, we have developed a rational guided peptide design workflow, applicable to both viral and non-viral vector optimization.

This protocol consists of the following key components:

- (i) Target Discovery: Identifying the receptor of interest based on its tissue expression and accessibility at the cell surface, setting the stage for precise therapeutic targeting.
- (ii) Receptor Analysis: Annotating the target receptor’s structure and potential binding partners and identifying essential binding hotspots, as well as examining the receptor’s behavior in a solvated environment. This analysis is crucial for understanding its interactions and functionality under physiological conditions.
- (iii) Ligand-Based Drug Discovery: Using both physical-based and data-driven methods, novel binders are designed to specifically target the identified receptor.
- (iv) Ligand Optimization and Vector Engineering: Evaluating the potential integration of the most promising peptides into AAV capsids, employing methods to restrain peptide conformations and incorporating the best candidates into AAV capsids. This approach not only enhances delivery efficiency but also maximizes the therapeutic potential of the developed compounds.

We focus particularly on the peptide modeling approach, highlighting its importance in enhancing the targeting ability of AAV vectors. We extended an initial database of several hundred binders to millions of potential ligands. We then prioritized promising peptides based on two criteria (i) their capabilities to still interact with the receptor while inserted in the capsid and (ii) their predicted free binding energy against the receptor. This approach led to the identification of the most promising candidates. The best ones will be then inserted in the capsid and the binding mode of the capsid-receptor complexes will be analyzed. This rational design workflow holds

promise for advancing gene therapy applications by improving the precision and efficacy of AAV-mediated gene delivery.

P0066

Advanced analytical methods optimize rAAV process development outcomes and improve yields and full particle ratios

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As the leading vehicle of gene therapy for treating several human diseases, there has been a rapid increase in the efforts to manufacture rAAVs on a large scale. Nevertheless, developing gene therapy manufacturing that delivers both quantity and quality at a commercial scale remains a highly complex endeavor. The biggest challenge of rAAV production is to minimize the number of empty particles that lack the genome of interest while delivering the highest yield of full capsids. Thus, accurately determining the percentage of full particles is critical for the final product and at each step of the purification to support process optimization. Mass photometry (MP) has emerged as a rapid and accurate biophysical method to measure the mass distribution and heterogeneity of rAAV preparations. By measuring the light scattering of single particles as they adsorb onto a glass surface, MP can distinguish between empty, partial, and full rAAVs due to their difference in mass. Here, we report our approach of using MP, to evaluate several critical process parameters, select the most suitable purification conditions, and adjust the process for optimal performance. First, we evaluated selected upstream production parameters of a triple transfection process for AAV5. In addition to the Rep/Cap and GOI plasmids, HEK293 cells grown in suspension were transfected with Helper Plasmid A or Helper Plasmid B. To identify the optimal stoichiometry, cells were transfected with two different plasmid ratios. After an optimized clarification, characterization by MP showed that cells transfected with Plasmid B and ratio 1 produced the highest proportion of full rAAV particles (16.5%) compared to ratio 2 (8.2%). Cells transfected with Plasmid A showed on average 8% full particles for both ratios. Further downstream purification was carried out by anion exchange chromatography. Here, MP was used to monitor the empty-to-full ratio and the impurities of four elution fractions (E1-E4). From a load material with 18.5% full capsids, elutions E2 and E3 were the most enriched in full particles with 31% and 28.1%, respectively. Although E4 showed a small population of full particles (11.1%), MP revealed the presence of significant lower-mass impurities. By rapid at-line monitoring of the % full AAV, the process development team can quickly decide which elution fractions to pool for further processing, troubleshooting with additional analytics when necessary or, if faulty, stop the process saving time and resources. To further understand genome heterogeneity, Nanopore Sequencing was used to confirm the identity of two rAAV preparations bearing the same GOI but produced with different Helper and RepCap plasmids. Using our in-house bioinformatics pipeline, we found that rAAV-A had significantly lower DNA contamination compared to rAAV-B. Specifically, rAAV-A had 1.8% from backbone packaging, 1.2% from RepCap, 0.4% from Helper, and 5.5% from host cell DNA, whereas rAAV-B had 8.4%, 4.8%, 1.7%, and 19.7%, respectively. Our findings underscore the importance of identifying the right analytical tools to understand and optimize each step of rAAV manufacturing and highlight how an agile integration of advanced analytics accelerates process development.

P0067

Assessing device compatibility through assay matrix approach ensures therapeutic consistency and patient welfare

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The study aimed to assess the impact of exposure time to the subretinal injection device on the drug product, a pivotal consideration for clinical trial efficacy and safety. The drug product was subjected to dilution following the investigator's brochure protocol to create sample sets representing potential low and high dosages, and exposure durations longer than clinically anticipated. Triplicates of each dosage were exposed to the sterile syringe and subretinal injection needle for 6hrs at ambient temperature or 30 minutes in cold storage, while control samples underwent identical preparation and storage conditions without exposure to the injection apparatus.

Assays were conducted to evaluate vector concentration, vector infectivity, expression, and potency. Results from these assays revealed no significant disparities in these crucial parameters between the exposed and non-exposed samples. Importantly, the observed differences fell within the margins of precision for each assay, indicating consistency and reliability. These findings remained consistent across both the low and high dosage sample sets.

The study's outcomes provide reassurance regarding the stability and integrity of drug product under conditions simulating clinically relevant exposure to the subretinal injection device. The absence of substantial effects on vector concentration, infectivity, expression and potency of the drug product suggests that exposure time to the injection apparatus within the specified duration does not compromise the therapeutic efficacy or safety profile of the drug in-vitro.

In conclusion, the study contributes valuable insights into the optimization of subretinal injection protocols for drug product administration, emphasizing the importance of meticulous attention to procedural details in ensuring therapeutic consistency and patient welfare. Future research endeavors can build upon these findings to refine administration techniques and enhance the clinical utility of the drug product in addressing the unmet needs of patients afflicted with retinal diseases.

P0068

Development and qualification of potency assay: guidelines and strategies

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Gene therapy products hold immense promise for treating various genetic disorders; however, ensuring the potency, efficacy, and safety of such therapies necessitates the development and qualification of robust potency assays. This poster delves into the meticulous process of developing and qualifying a potency assay, with a focus on strategies and guidelines.

The development phase commenced with a thorough optimization of the workflow. Parameters critical to assay performance, including seeding density, transduction time, and the range of multiplicity of infection (MOI), were systematically varied and evaluated. This optimization aimed to enhance assay sensitivity and reliability while minimizing variability.

Analysis and qualification of the potency assay involved a comprehensive assessment of pre-qualification and qualification data. Key performance parameters such as specificity, repeatability, intermediate precision, accuracy, linearity, and range of the test method were rigorously evaluated against predefined criteria outlined in the qualification protocol.

A critical aspect of the qualification process was the selection of appropriate reagents. Reagents play a pivotal role in assay performance and reliability. Careful consideration was given to the sourcing, characterization, and validation of reagents to ensure consistency and reproducibility across experiments.

Ultimately, the potency assay demonstrated robust performance across all critical parameters. Specificity assays confirmed the ability of the assay to accurately measure the potency without interference from other components. Repeatability and intermediate precision studies demonstrated the assays' reliability and consistency within and between experiments. Accuracy assessments confirmed the assays' ability to provide results close to the expected potency values. Linearity and range studies established the assay's ability to accurately measure potency over a broad range of concentrations.

In conclusion, the development and qualification of a potency assay by employing meticulous cell line engineering strategies and adhering to guidelines represent a significant milestone in ensuring the efficacy and safety of this gene therapy products.

P0069

Developing a comprehensive renal delivery platform for monogenic kidney disease targeting by exploring local and systemic routes

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Chronic kidney disease (CKD) is a growing public health challenge, affecting roughly 1 in 10 people globally. The main drivers are diabetes and hypertension. However, monogenic variants have recently been recognized as significant contributors. Once patients reach end-stage renal disease (ESRD), the only treatment options are dialysis or transplantation, which are associated with high morbidity and mortality. Advances in CRISPR-Cas genome editing offer promising alternative strategies to directly correct the underlying genetic variants. However, the kidney's complex anatomy and diverse cell populations present significant obstacles to the efficient and specific delivery of gene therapies. Current *in vivo* studies using local delivery indicate that certain serotypes of adeno-associated viruses (AAVs) can transduce renal cells, although with low efficiency and specificity to particular cell types. However, these studies often evaluate stable reporter gene expression, missing transient or low-level transduction crucial for gene editing. Utilizing an Ai14 reporter mouse model, where vector-mediated Cre recombinase delivery excises a loxP-flanked STOP cassette to induce tdTomato expression, allows to detect both high and low transgene expression from distinct delivery vectors.

Here, we used this model to explore and optimize kidney delivery by incorporating local and systemic delivery techniques, novel AAV serotypes and promoters, as well as non-viral methods such as lipid nanoparticles (LNPs).

Testing AAV8 and AAV9 serotypes via local renal pelvis, artery, and vein injections, as well as systemic tail vein injections, revealed route-dependent distribution patterns of tdTomato-expressing cells by immunofluorescence. While renal vein and tail vein injections resulted in tdTomato expression primarily in glomerular or adjacent tubular cells, renal artery and pelvis injections additionally showed signals in the medullary region of the kidney. Using flow cytometry analysis of whole organs, we quantified the exact proportion of transduced cells. In the liver, approximately 90% of cells were transduced, regardless of the injection route, compared to up to 6% kidney cell transduction with renal artery injections. No significant difference between AAV8 and AAV9 was observed regarding distribution pattern and the proportion of transduced cells.

For our non-viral approach, initial LNP formulations were generated using a microfluidic nanoparticle formulation system. *In vitro* transfection of eGFP resulted in 80% efficiency in HEK293T and HepG2 cells, while renal tubular epithelial cells showed only 0.7% transduction. By modifying the ionizable lipid in the formulation, LNPs transfected mouse immortalized renal tubular epithelial cells and primary human renal epithelial cells with up to 52% efficiency, as determined by flow cytometry.

Previous reports of renal delivery may not accurately reflect transduction efficiency due to the applied *in vivo*system, rarely explore local delivery and route-dependent distribution, and lack precise quantification of transduced cells by flow cytometry. Our reporter system facilitates robust and quantitative analysis of renal *in vivo* delivery, enabling systematic optimization of transduction efficiency. Ongoing experiments are exploring additional AAV serotypes in combination with cell-type-specific promoters to enhance specificity and efficiency, as well as optimising LNPs in advanced cell models for eventual *in vivo* applications.

P0070

Gene Therapy of Hepatic Encephalopathy with Adeno-Associated Virus

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Hepatic Encephalopathy (HE) is a chronic neuropsychiatric complication mainly present in patients with liver cirrhosis or hepatocellular carcinoma. It is characterised by various stages of motor and cognitive dysfunctions, and in some severe cases, it can progress to intracranial hypertension, cerebral oedema, and death. HE originates from impaired detoxification functions of the damaged liver in these patients, leading to elevated ammonia levels in circulation and the

brain. In previous studies, we developed the recombinant baculovirus Bac-GS to reduce plasma ammonia levels and alleviate HE. Bac-GS was inoculated into skeletal muscle (i.m.), where it mediates the expression of the glutamine synthetase enzyme, promoting the binding of ammonia to glutamate to synthesise glutamine. Gene therapy with Bac-GS has successfully reduced ammonium levels in rat models of acute and chronic hyperammonemia. However, Bac-GS-mediated GS protein expression is transient, making it useful only for controlling acute episodes of hyperammonemia and HE. In this study, we are evaluating the use of adeno-associated viruses carrying the GS transgene (AAV-GS) with the aim of developing a long-term effective therapy, considering that HE patients have an average survival rate of five years. The pseudotyped 2/8 AAV-GS was produced using the *Sf9* insect cell-baculovirus system and purified on an iodixanol gradient. In this novel approach, AAVs were tested in a rat model of chronic liver damage caused by the carcinogenic compound diethylnitrosamine (DEN). Using this toxic compound, we replicated the progression and stages of liver injury, such as fibrosis, cirrhosis, and hepatocellular carcinoma, as well as elevated ammonia levels in circulation. In the DEN-chronic liver damage model, we identified altered motor coordination in rats subjected to a rotarod test. However, the i.m. administration of AAV-GS significantly restored motor control in these rats. These results suggest that gene therapy with AAV-GS could alleviate HE in humans.

P0071

Design-of-experiment (DoE) approach suggests transfection viable-cell density as key parameter for optimized rAAV productivity and total DNA amount for rAAV packaging

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Transient triple-plasmid transfection of HEK293 cells is one of the main production methods employed for clinical and commercial recombinant adeno-associated virus (rAAV) manufacturing. There is currently a challenge to meet the rAAV demand for clinical trials, making it essential to enhance the yield per cell while obtaining the maximum amount of genome-containing capsids. We believe transfection efficiency is critical for increased rAAV productivity. The efficiency of gene delivery relies on physicochemical properties of the transfection components such as total DNA amount or transfection reagent to DNA ratio, and on cell physiology.

Response surface methodology (RSM) statistical model, using a DoE approach with JMP® statistical software, was selected to analyze the effect of two of the main factors on rAAV productivity and packaging: viable cell density (VCD) and total DNA amount. Titration showed that transfection VCD has a strong impact on productivity with negligible impact due to very low or high cell density, or total transfection DNA amounts. Conversely, a decrease in the amount of DNA during transfection correlates with an increase in the percentage of full capsids as measured by Analytical Ultracentrifugation (AUC) or Mass photometry (MP). DNA:PEI complex characterization indicated that increasing total DNA amounts led to greater complex size measured by Dynamic Light Scattering (DLS), which may suggest a decrease in transfection efficiency and therefore a lower packing capacity.

P0072

Prevalence of errors in lab-made plasmids across the globe

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Plasmids are indispensable in life sciences research and therapeutics development. Currently, most labs custom-build their plasmids. As yet, no systematic data on the quality of lab-made plasmids exist. Here, we report a broad survey of plasmids from academic and industrial labs worldwide. We show that nearly half of them contained design and/or sequence errors. For transfer plasmids used in making AAV vectors, which are widely used in gene therapy, about 40% carried mutations in ITR regions due to their inherent instability, which is influenced by flanking GC content. We also list genes difficult to clone into plasmid or package into virus due to their toxicity. Our finding raises serious concerns over the trustworthiness of lab-made plasmids, which parallels the underappreciated mycoplasma contamination and misidentified mammalian cell lines reported previously, and highlights the need for community-wide standards to uphold the quality of this ubiquitous reagent in research and medicine.

P0073

Plasmid engineering to improve AAV productivity and packaging efficiency

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The genome packaging efficiency (full to total capsid ratio, or F/T ratio) for Adeno-Associated Virus (AAV) is a critical quality attribute for the manufacturing and commercialization of AAV-based gene therapies. A higher F/T ratio has the potential for reducing dosage and eventually the cost of goods (COGS). In this study, we present design improvements in pHelper and pRepCap plasmids that can support increased AAV titers, whilst improving F/T ratio in upstream crude harvest using a triple-plasmid transfection process. Performance was measured both at Lonza and at BMS R&D labs independently. Both scenarios show significant increments in full ratio. A tentative plan to further improve productivity will also be shared.

In summary, this study demonstrates that we can significantly increase the AAV productivity and packaging efficiency for various AAV serotypes and client-specific Gene of Interest (GOIs) at the upstream production stage through plasmid engineering approaches.

P0074

The Advanced Ligand Conjugation (ALIGATER™) platform chemically redirects AAV and LNP vectors towards specific receptors via peptide-mediated targeting

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Because of their non-specific tissue tropism, current gene therapy vectors necessitate high doses that increase manufacturing costs and elevate the risk for adverse side effects. In recent decades, several techniques have been developed to enhance vector tropism, notably that of AAVs, the most widely used vector for gene therapy. The most common approach aims at manipulating the interactions between the AAV capsid proteins and their cell attachment factors/receptors. To do so, new vector binding sites can be genetically engineered through the insertion of novel peptides within the capsid's exposed loops. While effective, this approach has notable limitations, including constraints on the number, size, location, and presentation of these binding sites, as well as the necessity to redesign them for each new serotype.

An alternative strategy applicable to both viral and non-viral vectors is to chemically conjugate targeting ligands at the vector surface. Typically, this is achieved through a 2-step process implying prior modification of the capsid to introduce an anchor element for subsequent ligand attachment. Although this method offers greater design flexibility, it also introduces significant complexity into vector preparation and can negatively impact manufacturability as well as capsid assembly. Coave's proprietary ALIGATER™ platform addresses these challenges by employing a one-step chemical conjugation process. This approach enhances AAV vectors without the need for prior surface modification, making it compatible with virtually all existing natural or engineered AAV serotypes. Moreover, this conjugation is seamlessly integrated into the downstream processing of AAV manufacturing, preserving both existing processes and yields. Our first-generation of conjugated AAVs (coAAV), utilizing sugar-based ligands to enhance diffusion and tissue distribution, have demonstrated superior performance compared to benchmark vectors in the CNS and the retina of mice, rats and non-human primates. In this work, we extend the ALIGATER platform's utility by demonstrating for the first time its ability to functionalize AAV variants with peptide ligands for precise retargeting of vectors to specific human receptors.

To illustrate the platform's flexibility, we conjugated several AAV serotypes with various peptide-based ligands targeting receptors, such as TfR1 or integrins, and modulated their density per capsid. We showed that coAAV2 and coAAV9 vectors can be effectively retargeted to cells expressing the receptor of interest. The targeting was dependent on ligand density and could be blocked in competition assays. Additionally, the same ligands were successfully conjugated to LNPs post-formulation, highlighting the flexibility and versatility of Coave's proprietary chemistry and demonstrating ALIGATER™'s broad applicability. These peptide-conjugated vectors are currently being evaluated *in vivo* in mice expressing the human receptors. ALIGATER™'s simplicity, modularity, and compatibility with existing manufacturing processes enable efficient enhancement of gene therapy vector specificity at minimal cost, with strong prospects for clinical development.

P0075

Advancing adeno-associated virus vector manufacturing: a comparative study of transient transfection and stable producer cell lines

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Current processes for manufacturing adeno-associated virus (rAAV)-based vectors often involve transient co-transfection of multiple plasmids into mammalian suspension cell lines, providing all necessary genetic components but at high costs and with process variability. Recent progress in producer cell lines, housing all vector production genes within the cell genome, offer potential for enhancing AAV manufacturing. A collaboration between Spark, Roche, and Cytiva has yielded stable, high-titer producer cell lines for AAV vectors featuring clinically relevant transgenes, however, limited comparative data exists between vectors produced via transient transfection and those from producer cell lines. This study comprehensively compared AAV vector quality from transient transfection and producer cell line processes across bioreactor operation modes and cell culture scales. The analysis included genome packaging, capsid protein ratios, vector potency, and residual impurity measurement to capture process variability. Insights gained will inform how process-related changes may affect vector quality, potentially guiding the future integration of stable producer cell lines into clinical strategies, supporting consistent delivery of high-quality AAV vector products to patients.

P0076

Comparison of AAV downstream purification process using chromatography and density gradient ultracentrifugation

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1: Merck Millipore

Recombinant adeno-associated viral (rAAV) vectors provide an efficient and effective tool for therapeutic gene delivery. During genome packaging in host cells, a heterogeneous population of vectors is produced consisting of full, empty, and partially full capsids. An excess of empty capsids in a therapeutic dose can trigger unintended immune responses; therefore, therapeutic potency requires the removal of empty capsids for final formulation.

Empty and full capsids are similar in their physical characteristics and vary slightly in isoelectric point (pI) and density which makes separation challenging. Here we compare two methods for the separation of empty and full capsids based on the mentioned characteristics. A cesium chloride (CsCl) density gradient ultracentrifugation (DGUC), that is AAV-serotype independent and a chromatographic method utilizing AEX resins. We demonstrate the capabilities, efficiencies, and challenges of two methods using models AAV2 and AAV5 in downstream vector processing. Both methods are evaluated from similar start material using affinity capture chromatography feed. Our findings demonstrate the deeper understanding and importance of the two methods in

terms of their usage depending on the requisites of the final formulation. Each method provides flexibility based on the therapeutic drug product's production scale, availability of instruments, raw materials, and technical expertise.

P0077

Reimagining cell lines for AAV manufacturing

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Over the past decade, gene therapy has revolutionized the treatment of inherited diseases. Key to this success are the recombinant adeno-associated virus (AAV) vectors used extensively for transgene delivery in clinical studies and approved applications. However, as focus turns from rare diseases to more prevalent indications, a major challenge is establishing production processes that can respond to the increasing demand for large quantities of high-quality AAV.

Stable production methods are well-established in large-scale monoclonal antibody (mAb) manufacturing. A similar approach can be used for AAV, but creating such a cell line is complicated by the fact that four genes must be incorporated to generate a true producer cell line. Cytiva has leveraged its expertise in cell line development, in-depth understanding of rAAV basic biology, and long-standing experience in upstream process development to do just that.

ELEVECTA™ producer cell lines enable high yield, scalable, and robust manufacturing processes. All components necessary for AAV vector production are stably integrated into the genome of the cell line, eliminating the need to use transient transfection or helper virus.

A further focus area for the industry is vector quality, as tackling impurities is a major chemistry, manufacturing, and controls (CMC) challenge in current AAV manufacturing processes. Unpackaged host cell components like DNA, RNA, proteins and lipids (collectively known as “process-related impurities”) can be removed by conventional downstream methods. However, product-related impurities, which involve host cell components that are incorporated in the viral capsid, are intrinsically resistant to classic purification methods.

Among these encapsidated impurities, host cell-derived DNA (hcDNA) represents the most problematic stowaway. It has been reported that in purified preparations from mammalian producer cells, 1% to 3% of all genome-containing AAV particles still carry encapsidasted hcDNA fragments. Safety concerns associated with encapsidated hcDNA could be related to potential genotoxicity or immunotoxicity. An example of the former would be insertional mutagenesis or packaging of an oncogene into AAV particles, whereas the latter might result from adverse immune responses that can lead to inflammation. For these reasons, production methods that minimize encapsidated hcDNA would be a beneficial update to current AAV manufacturing processes.

P0078

Ensuring gene therapy product quality through advanced characterization analysis with TEM

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1: QuTEM

Ensuring the highest product quality during all stages of product development and its life cycle is key to ensuring the safety and efficacy of medicinal products. Robust characterization analyses, providing precise data on critical quality attributes, are essential. Transmission Electron Microscopy (TEM) serves as a powerful tool for investigating the microscopic world of gene therapy vectors, such as Adeno-Associated Virus (AAV) and lentiviral vectors (LVV) products. While TEM has traditionally been recognized for delivering qualitative visual data, QuTEM's advancements in image analysis have enabled the objective and accurate translation of intricate visual details into critical quality attributes. This innovation is vital for understanding product efficacy, safety, and stability over time.

P0079

Optimized plasmid backbones for efficient rAAV manufacturing

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Advanced therapies aim to treat uncurable diseases by providing the cells in a patient's body with the capability to synthesize an active ingredient by itself. To do so, they provide a genetic blueprint that governs the expression of a molecular target. Recombinant adeno associated virus (rAAV) is one of the most intensively studied and most widely used vector to mediate the delivery of this genetic information. To efficiently deliver cargo to the recipient cells, high vector doses are necessary. Plasmid DNA is a critical raw material for manufacturing of rAAV in packaging cell lines. The virus titers that can be produced is critically dependent on the number of cells that are transfected by the Helper-, Packaging- and Transfer plasmids and by their biological activity within the cell. Accordingly, both the development and application of advanced therapies demand for optimized raw materials in the form of plasmids that exhibit high biological activity and can be produced both at high qualitiy and at high titers. The availability and thus the affordability of these raw materials in turn demands for high performing production processes. Plasmid DNA is produced in a fermentative process using bacteria such as *Escherichia coli*. High performing production processes are characterized in that they provide high product titers, allow the isolation of highly pure plasmid DNA, and allow scalability from development to GMP scale. All these characteristics can be influenced by specific sequences on the target plasmid, especially those that are crucial for functionality and efficiency of rAAV. To mitigate these concerns WACKER developed a proprietary fed-batch process that optimally supports the growth of PLASMITEC® strains and allows optimal plasmid replication. This process allows the scalable production and isolation of plasmid DNA – including critical raw materials for rAAV manufacturing – at high specific titers and at high purity. Using this process, we developed specific DNA sequences that further improve productivity of the target plasmids and thereby

reduce the cost of manufacturing. In parallel, we screened for alternative plasmid architectures that increase both their transfection efficiency and their biological activity in packaging cell lines. In combining the resulting technologies, we developed proprietary plasmids that facilitate rAAV manufacturing by providing both more accessible and more efficient raw materials. With its productivity, flexibility and scalability, the PLASMITEC® manufacturing platform aims to provide high quality and affordable raw materials and is therefore an invaluable facilitator for the development and application of advanced therapies.

P0080

High-throughput affinity purification for AAV vectors: From microscale optimization to bench-scale production

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Adeno-associated virus (AAV) vectors are essential in gene therapy due to their stability, scalability, and minimal immune response. Integrating automated microscale bioreactors, such as the Ambr® 15 system, has revolutionized early-stage upstream process optimization. However, the progress of gene therapy requires the development of a corresponding microscale affinity purification process. This would enable batch processing of the Upstream samples to better analyze the quality of AAV vectors, e.g. - measuring the percentage of full capsids, as well as measure titer. This process can be carried out using automated systems to ensure reproducibility and consistent results. Moreover, it enables a Design of Experiments (DOE) based approach for the screening of chromatography parameters. This presentation outlines a high-throughput affinity purification approach that can be applied for any AAV serotypes, from microscale experimentation to bench-scale optimization.

P0081

Enhanced ITR Stability and Production Efficiency with PowerS™-ITRrs strain in Gene Therapy Development

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Adeno-associated virus (AAV) is increasingly recognized as a powerful tool for gene transfer and genome editing applications in gene therapy. Its effectiveness hinges on two short inverted terminal repeats (ITRs) flanking the AAV genome. These ITRs contain essential cis-acting elements necessary for the rescue, replication, and packaging of the genome (refer to Fig. 1). However, the structural instability of ITRs often leads to deletions during the propagation of AAV transfer plasmids. This results in reduced plasmid yields, lower viral titers, decreased packaging efficiency, and increased variability in downstream process. Addressing these challenges is crucial; therefore, developing a stable host strain and optimizing the production process for manufacturing AAV transfer plasmids is imperative. Our focus on enhancing the stability of ITRs and refining

manufacturing techniques ensures high-quality, efficient production of AAV vectors, paving the way for reliable and groundbreaking advancements in gene therapy.

P0082

Preliminary safety and efficacy data of a phase 1/2 clinical trial to support the use of high dose intrathecal AAV9/AP4M1 for the treatment of patients with Hereditary Spastic Paraplegia Type disease

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Hereditary Spastic Paraplegia Type 50 (SPG50) is a rare, childhood-onset autosomal-recessive disease caused by bi-allelic loss-of-function variants in the *AP4M1* gene, which impacts adaptor protein complex 4 function. This results in a progressive neurological syndrome characterized by progressive spastic paraplegia, global developmental delay, secondary microcephaly and seizures, with onset typically in early childhood. The majority of children become non-ambulatory and full-time wheelchair users.

We present preliminary safety data of four patients, up to 24 months post treatment in a phase 1/2 clinical trial using targeted gene replacement of the AP4M1 gene across the CNS. No deleterious immune responses have been noted, and the treatment has been tolerated well across all 4 subjects. There has been no evidence of DRG toxicity and nerve conduction has been shown to be stable or improved following gene transfer.

Our recombinant vector is designed to achieve life-long expression of AP4M1 and broad CNS transgene distribution. Our approach utilizes early intervention and higher intrathecal AAV doses than are used in most other intrathecal AAV clinical trials. Doses given to 3 subjects aged 3-5 years were $1x10^{15}$ vg (total dose per patient), and $4x10^{14}$ vg to a 5-month-old presymptomatic infant. SPG50 treatment is challenging as it does not benefit from cross correction or amenable to enzyme replacement therapy. We speculate that the self-complementary design of the vector contributes to its potential efficacy, and the relatively modest strength UsP promoter contributes to its safety. Immune management was designed to allow inclusion of both CRIM (-) and CRIM(+) patients, also thought to be contributing to the overall safety of patients. Interim data from the trial, including efficacy outcomes, will be presented.

Disclosure:

Members of the research team, Dr. Steven Gray and Dr. Xin Chen, are inventors of the AAV9/AP4M1 product being evaluated in this study. Depending on the outcome of this study, Drs. Gray and Chen may be entitled to licensing, royalty, or other payments. Additionally, Dr. Gray's laboratory is conducting one of the several tests on the AAV9/AP4M1 product to determine its suitability to be used in this study, as per FDA requirements. In addition, UTSouthwestern Medical Center has the potential to receive future benefits based on the outcome of this trial.

P0083

Impact of enhancing agent on recombinant adeno-associated virus 5 (rAAV5) vector production

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1: Merck Millipore

Recombinant adeno-associated virus (rAAV) has established itself as a highly successful gene delivery vector with a well-characterized safety profile allowing extensive clinical application. Recent successes in rAAV-mediated gene therapy clinical trials will continue to drive demand for efficient rAAV production processes to reduce costs. Here, we demonstrate that small molecule bioactive chemical additives and small peptides can significantly increase recombinant rAAV5 vector production by human embryonic kidney (HEK) cells. Among the class of enhancers evaluated were microtubule inhibitors, histone deacetylase (HDACs) inhibitors, and small peptides with caspase inhibitor activity. In addition to these, we evaluated the Mirus Bio enhancer known to demonstrate improvement in rAAV titers. The evaluation was based on both genomic and capsid titers.

We used a DOE (Design of Experiment) approach to design and experiment for screening enhancers using 48 Ambr® 15 vessel bioreactor vessels. The data generated helps us select the best-enhancing agent and concentrations. These selected enhancers will be evaluated at different scales and bioreactor types for confirmation.

We demonstrated that small molecule enhancers of rAAV5 production are amenable to optimization in an existing suspension HEK293 cell system and that positive hits from the initial small-scale screening of enhancer molecules are translatable to improvements in genome titer, with the potential for translation to commercially viable volumetric production.

P0084

Manufacturing platform for high-quality AAV starting plasmids

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Gene therapy vectors based on the adeno-associated virus (AAV) have emerged as one of the most preferred choices in approved applications and clinical trials. Plasmid DNA is an important starting material for manufacturing AAV virus particles. Frequently, a set of three plasmids is applied, such as the plasmid coding for the recombinant transgene, the packaging plasmid coding for the AAV capsid (Rep/Cap) and finally the adenovirus (Ad) helper plasmid. Supply of the respective AAV starting plasmids in high quality and quantity has become a bottleneck in the manufacturing of AAV gene therapies, posing a considerable challenge for plasmid manufacturers.

Biomay has established a proprietary production process for AAV plasmids manufactured under the quality standard of Good Manufacturing Practice (GMP). For manufacturing, an *E. coli* master cell bank is generated, which is then used in a high cell-density fed-batch fermentation process to propagate the plasmid. After harvesting and alkaline lysis of the cell mass, the product is purified

in a two-step chromatography process and formulated by ultra/diafiltration. Finally, aseptic filling using an automated filling line is performed to finish the plasmid product.

The manufacturing process steps (fermentation, cell lysis, chromatographic purification) have been optimized to cover the most relevant AAV packaging plasmids (Rep/Cap serotypes) as well as the large adenovirus helper plasmids at appropriate quality, high purity and economic yield. Our results also show that the process can accommodate different *E. coli* host strains with comparable outcomes. Good scalability of the process was confirmed over a wide scale range (from milligrams over multiple grams up to ~100 g). Plasmid quality was characterized by a comprehensive set of analytical methods. Particular attention was paid to confirming the identity and integrity of repetitive DNA elements. Our data show high plasmid homogeneity (>90 % of supercoiled conformation), low host-derived impurity levels and high batch-to-batch consistency across all AAV plasmids manufactured.

In addition to client specific development and manufacturing (CDMO) services, a concept has been established to provide pre-manufactured and ready-to-use AAV starting plasmids in GMP quality on an off-the-shelf basis. The set of Rep/Cap plasmids include the major capsid serotypes used in current clinical studies, such as AAV2, 5, 6, 8, and 9. Moreover, an optimized Ad helper plasmid with minimized size was manufactured. Suitability of the vectors for AAV virus particle manufacturing was confirmed using HEK293 cell culture and detection by qPCR and ELISA.

With the established platform, more than 20 different AAV starting plasmids have been manufactured under GMP conditions to date.

P0085

Enabling Commercial AAV Manufacturing by Helper Virus-free, Suspension HEK293-based Xcite® AAV Stable Producer Cell Lines

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Current AAV manufacturing often suffers from low productivity and scalability, high cost of raw materials, supply chain complexity, and high batch-to-batch variation. To overcome these limitations and industrialize AAV manufacturing, we have developed high-performing, helper virus-free, Xcite® AAV stable producer cell lines (PCL). The cell line is built upon our proprietary high-producer HEK293 cells, grown in chemically defined, serum-free, ADCF medium in suspension. The DNA expression vectors are designed to tightly control cytotoxic viral gene expression via engineered TET-inducible promoters, and stably integrated into the cell genome in one step by our VS *piggyBac*™ transposon technology. Upon induction at regular cell density, the selected PCL clones outperform transient transfection process with higher AAV titer (>1E12 vg/mL) with comparable AAV full/empty capsid ratio (>30% full capsids) and infectivity at harvest. Moreover, these cells maintain long-term stability (> 30 passages) for AAV productivity and packaging efficiency. Helper-virus free AAV PCL eliminates the need for costly plasmids, reduces supply chain and manufacturing complexities and can be easily scaled to 2000L+. Taken together, our Xcite® AAV platforms can enable high quantity and quality of commercial AAV manufacturing at a scale you need for your valuable therapies.

P0086

Extended characterization of AMT-191, an AAV gene therapy for Fabry disease

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Fabry disease is an X-linked genetic disorder, caused by mutations in the α-galactosidase A (GLA) gene. AMT-191 is a gene therapy product, produced using the baculovirus expression vector system (BEVS), that delivers a GLA transgene to target the liver to produce GLA protein. To gain in-depth understanding of AMT-191 product characteristics, extended characterization was performed across a subset of representative AMT-191 batches. In these studies, the protein component including post-translational modifications (PTMs), DNA component, and higher order structure were examined using multiple orthogonal methods.

The identity and integrity of the protein component of AMT-191 was confirmed by Western blot. In addition, SDS-PAGE followed by Silver stain or SYPRO Ruby showed that VP1, VP2, and VP3 are the main protein components. The identity was further confirmed using peptide mapping, showing >97% coverage of the reference capsid protein sequence. Intact mass spectrometry analysis demonstrated that the observed molecular masses of VP1, VP2, and VP3 were as expected, based on the capsid sequence. Post-translation modification (PTM) analysis showed that acetylation, methylation, and di-methylation were the most prominent modifications, which was consistent between tested batches. Low level deamidation was also observed for all batches. Neither glycosylation nor phosphorylation was observed.

The DNA component was assessed by gel electrophoresis using neutral and denaturing conditions. The results revealed the presence of a major band at ~2.1 kb, corresponding to a single copy of the expected vector genome sequence. Additional bands were observed that were twice the length (~4.1 kb) and between ~2.1 and ~4.1 kb. NGS analysis confirmed the vector genome sequence, and no mutations were observed in the GLA expression cassette (containing the promoter, transgene, and polyA sequence). The results for the AMT-191 DNA component were highly similar for all tested batches.

The analysis of the higher order structure and composition of AMT-191 confirmed the expected combination of empty, partially filled, and full particles, and a largely monomeric particle population with particles of expected size. The results of SV-AUC, SEC-MALS, and cryo-TEM consistently demonstrated that the particle profile of the tested AMT-191 batches mainly consists of a large fraction of capsids containing at least the size of, and up to two times, the intended vector DNA, and a smaller fraction of empty and partially filled capsids. SEC-MALS also showed that the tested batches are consistent in purity, showing a >99.2% relative main peak area, representing single (monodisperse) AAV.

Overall, the extended characterization studies confirmed the identity of the protein and DNA components for all tested batches. Using multiple orthogonal methods, it was demonstrated that AMT-191 batches contain a small fraction of empty and partially filled capsids, with the majority of the capsids containing the expected vector genome. The tested AMT-191 batches show overall a high level of consistency regarding the extended characterization results.

P0087

Optimizing Intracranial Gene Delivery into Brain Parenchyma with a Next-Generation AAV5-Based Capsid Variant

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Gene therapies for brain disorders offer considerable promise; however, they face substantial hurdles in effectively accessing the brain parenchyma due to inherent physiological barriers. In response to this challenge, intracranial delivery into the brain parenchyma has emerged as a logical strategy, bypassing these barriers to directly target sites with minimal loss of therapeutic material and reduced risk of off-target effects.

Central to intracranial-based gene delivery are adeno-associated virus (AAV) vectors, which play a pivotal role in facilitating safe and sustained therapeutic transgene expression after one-time administration. Among the diverse array of natural capsid serotypes, AAV5 has distinguished itself through its consistent safety profile, low immunogenicity, and promising efficacy in recent clinical trials. These qualities position AAV5 as one of the leading capsids for therapeutic gene delivery in brain disorders.

Building upon the promising profile of AAV5, a novel AAV5-based capsid variant has been developed and characterized. It has demonstrated enhanced transduction efficiency from intracranial delivery in rodent, minipig, and non-human primate (NHP) models, accompanied by reduced innate immune activation. In the NHP study, this next-generation AAV5-based capsid not only shows improved efficacy but also is capable of potently delivering therapeutic transgenes at approximately tenfold lower doses than its predecessor. These findings highlight the transformative potential of this capsid for central nervous system gene therapy in patients.

P0088

Development of a quantitative alpha-dystroglycan glycosylation test in patients with Limb Girdle Muscular Dystrophy R9 treated in ATA-001-FKRP open-label multicenter AAV trial

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Limb Girdle Muscular Dystrophy R9 (LGRMDR9) (former known as LGMD2I) is a rare recessive genetic myopathy leading to slowly progressing muscular weakness affecting skeletal, respiratory

and cardiac muscles, with a large spectrum of severities. The pathology is caused by mutations in the FuKutin-Related Protein (FKRP) gene, encoding a Golgi-apparatus membrane-bound glycosyl-transferase involved in the o-linked glycosylation of alpha-dystroglycan (αDG). αDG is a pivotal member of the Dystrophin-Associated-Protein-Complex, a multi-protein complex linking the extracellular matrix to the internal cytoskeleton. αDG plays a crucial role in the resistance of myofibers to contraction-induced injury during muscle contraction. ATA-001-FKRP (NCT05224505) is a Phase 1-2 open-label multicenter AAV gene therapy trial aiming at assessing the therapeutic efficacy of intravenous delivery of a functional *Fkrp* coding sequence in LGMRD9 patients. The therapeutic product is expected to restore αDG glycosylation, hence protecting the link between the extracellular matrix and the internal cytoskeleton. To assess the efficacy of gene therapy, we developed and characterized in-depth western-blot assay for measuring glycosylated-αDG levels. The specificity, reproducibility, and repeatability of the assay, assessed by 3 operators on healthy and patient donor muscles' proteins, showed this method to be robust and hence relevant as a clinical endpoint. Analyses performed on patients included in ATA-001-FKRP study showed that levels of αDG glycosylation increase globally consistently with the immunohistological results performed on the same samples. This assay is a major asset for the assessment of therapeutic efficacy in this trial and could be useful for other dystroglycanopathies, a large subset of muscle disorders.

P0089

The vMiX™ platform: Tailored miRNA gene therapy targeting molecular drivers of ALS and FTD

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Amyotrophic Lateral Sclerosis (ALS) and Frontotemporal Dementia (FTD) are devastating neurodegenerative diseases linked by common underlying genetics and pathology involving toxic cytoplasmic aggregates of RNA binding protein TDP43 and mutant SOD1 protein.

The common pathological feature in most ALS (95%) and a significant number of FTD cases (45%) is the presence of toxic TDP43 aggregates. TDP43 is a predominantly nuclear RNA binding protein crucial for RNA metabolism. Another RNA processing protein, Ataxin-2 (*ATXN2*), is one of modifiers of TDP43 toxicity. Research has shown that reducing *ATXN2* mRNA levels offers protective effects in models of TDP43 pathology.

We describe the development of vMiX™, an adeno-associated virus (AAV) vector platform capable of expressing proprietary microRNAs (miRNAs) to downregulate genes, including ATXN2 or SOD1 mRNA as a potential therapy for ALS and FTD. Others have shown that reducing levels of these targets is protective in disease models.

To generate precise miRNAs capable of potently silencing target genes, we designed multiple miRNA candidates against *ATXN2* and *SOD1* transcripts. Using next-generation sequencing, we thoroughly characterised: first, the cleavage precision of guide/passenger RNA and their strand ratio (the precursor miRNAs are processed into a duplex small RNA, with the guide RNA strand, which is loaded into the silencing complex (RISC) and the unstable passenger RNA; second, the silencing efficacy towards target mRNAs; and third, the impact on endogenous miRNA repertoire.

We demonstrated species-specificity through parallel studies in multiple in vitro and in vivo models. Combining rational design with functional screening, we optimised the vMiX™ AAV platform to achieve nucleotide-level specificity for robust target silencing while minimising off-target effects. The vMiX™ AAV platform provides a versatile, tailor-made miRNA gene therapy approach targeting TDP43 indirectly by reducing ATXN2 levels and directly to SOD1 mRNA in ALS-FTD. This miRNA technology aims to enable the development of powerful new treatment strategies for these devastating neurodegenerative diseases.

P0090

Orthotopic knock-in of a codon-optimized cDNA functionally corrects IL10RA-deficiency in iPSC-derived macrophages

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A subgroup of patients with inflammatory bowel diseases (IBD) suffer from monogenic inborn errors of immunity. Our group has reported genetic defects in IL10R and pioneered the use of allogeneic hematopoietic stem cell transplantation (HSCT) as a definitive cure for these patients. Even though HSCT has a success rate of about 80%, it remains associated with risks related to conditioning regimen toxicities, non-engraftment or graft rejection, and graft-versus-host disease. Here, we employed a CRISPR-Cas9/AAV6-mediated genome editing approach in vitro as an experimental correction strategy for IL10RA-deficiency.

To model IL10RA deficiency in induced pluripotent stem cells (iPSCs), we first disrupted the IL10RA gene by CRSIPR-Cas9 genome engineering. To restore IL10RA expression in IL10RA deficient iPSCs, a fully codon-optimized IL10RA transgene, along with a constitutively expressed tNGFR cassette, was inserted downstream of the start codon of the IL10RA gene using the Cas9/AAV6 system. We achieved very efficient (up to 95%) bi-allelic editing frequencies of the codon optimized IL10RA cDNA into the IL10RA genetic locus. To assess whether the IL10RA gene correction is capable to functionally restore IL10RA-dependent signaling pathways in IL10RA deficient cells, we differentiated control (WT), IL10RA-knock-out (KO), and IL10RA-corrected iPSCs into macrophages using an *in vitro* differentiation protocol. Our flow cytometric analysis of iPSC-derived cells confirmed efficient differentiation into mature macrophages (CD11b+/CD14+/CD45+) across all analyzed genotypes. In IL10RA-KO iPSC-derived macrophages, STAT3 phosphorylation (Tyr705) was abolished upon IL-10 stimulation. Importantly, phosphorylated STAT3 was reconstituted in IL10RA-corrected iPSC-derived macrophages after IL-10 stimulation. As expected, SOCS3 mRNA levels increased in unmodified and IL10RA-corrected iPSC-derived macrophages following IL-10 stimulation but remained unchanged in IL10RA-KO iPSC-derived control macrophages. Furthermore, IL-10 was not able to attenuate LPS-induced secretion of IL-6 and TNF-α in IL10RA-KO iPSC-derived macrophages. Notably, the anti-inflammatory response was re-established in IL10RA-corrected iPSC-derived macrophages.

In conclusion, orthotopic insertion of a codon optimized cDNA effectively restored IL10RA function in iPSC-derived macrophages, offering a novel approach for treating monogenic causes of IBD using advanced genome editing methods.

P0091

Successful capture chromatography DoE studies conducted for a range of AAV serotypes to reduce manufacturing costs and accelerate development

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AAV is nowadays the most widely used virus for in vivo gene therapy. To achieve high vector potency in specific target cells or organs, to reduce off target transduction and to overcome immunological barriers in AAV gene therapy, many naturally occurring AAV serotypes and isolates can be chosen. Further, in the past about 2 decades, a large number of capsid engineering technologies have been developed to further tune AAV capsids for a desired application. Capsid choice can impact both, upstream and downstream manufacturing of AAV vectors and necessitates capsid specific assay development. Starting material design and transfection optimization are the main parameters to be considered in upstream. For downstream processing, capsid specific adaptions are crucial in each process step starting from capture where affinity chromatography is commonly used. Here we show for serotypes AAV2, AAV5 and AAV8 how product recovery and vector quality are affected by varying the elution buffer conditions of the affinity step. For each serotype, the best elution pH and conductivity were determined using a design of experiment (DoE) approach. The capsid load was found to be non-significant within the tested range. In addition, the possible effects of the optimal affinity elution buffer conditions were evaluated with respect to a subsequent full empty polishing step and compatibility was confirmed. It was found that AAV5 behaved very differently compared to the other serotypes in terms of elution conditions. Finally, selecting an affinity resin that allows a wide dynamic range for capsid loading with a high affinity has shown to be key to reducing manufacturing costs and developing a robust capture step to provide safe vectors at reduced manufacturing costs.

P0092

Production and Physicochemical Properties of a Novel Cardiotropic and Liver-Detargeted AAV Capsid with Low Pre-Existing Immunity

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Adeno-Associated Viral (AAV) vectors represent a promising approach in the treatment of inherited and acquired cardiac disorders. However, pre-existing immunity and hepatotoxicity risks associated with current vectors have hindered their clinical efficacy. To address these challenges, we have previously screened a large library of natural AAV capsids to identify BMRN_AAVcap7, a capsid with low pre-existing immunity in humans and preferential cardiac-targeting and liver-detargeting in rodents and non-human primates (NHPs).

In this study, we evaluated production and physicochemical properties of recombinant BMRN_AAVcap7. AAV vectors were produced in an insect cell platform, achieving robust production titers with high full-to-empty capsid ratios. Furthermore, we demonstrate that BMRN_AAVcap7 can be efficiently purified using affinity chromatography followed by gradient density ultracentrifugation, yielding high recoveries across the different research process scales. Compared to a benchmarked capsid, BMRN_AAVcap7 vectors exhibit favorable impurity profiles, high transgene packaging efficiency, low vector aggregation, moderate thermal stability, and high content of viral proteins 1 and 2.

Additionally, we show that the affinity chromatography-purified BMRN_AAVcap7 contains distinct populations of full capsids that can be separated by gradient density ultracentrifugation and quantified using a fast protein liquid chromatography system (FPLC) with inline UV detection. Characterization of these distinct capsid populations was performed for multiple vectors with varying vector genome lengths and sequence, revealing differences in packaging, stability, and viral protein content. We also evaluated the relative in vitro potency of these populations in human cell lines to better understand which physiochemical properties of this novel capsid contribute to increased transduction.

In summary, our data shows that BMRN_AAVcap7 possesses favorable production and physicochemical characteristics, positioning it as a compelling AAV candidate for further exploration in cardiac gene therapy. More broadly, our findings suggest that natural capsids may complement engineered capsids in the toolbox of next-generation vectors for viral gene therapy.

P0093

Large-scale production of AAV vectors using fixed-bed bioreactor and suspension cell culture systems

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Efficient mass production of adeno-associated virus (AAV) vectors is essential to meet the rising demand for clinical trials and therapeutic applications. As the field of gene therapy expands, scalable methods for AAV vector production become increasingly necessary. To address this need, we have developed methods for the large-scale production of AAV vectors using fixed-bed bioreactor systems and suspension culture systems. In this study, we conducted a comparative analysis of these two methods and discussed considerations for optimizing the processes. Initially, we optimized AAV production using the fixed-bed bioreactor and suspension culture system, achieving productivity comparable to traditional 2D cell culture plates. We then compared the infectivity of AAV vectors produced by each method through flow cytometry analysis. We confirmed that AAVs produced by our large-scale production method have similar transduction efficiencies to those produced by the cell culture method. Our optimized protocols for AAV vector production are believed to be effective even when scaled up further. In conclusion, both of our AAV production methods offer high productivity and infectivity levels. These results indicate that these methods provide a reliable and adaptable strategy for producing high-quality AAV vectors suitable for diverse clinical applications in gene therapy. Through this comprehensive analysis of the fixed-bed and suspension culture systems, we anticipate that we can ultimately derive the optimal AAV production strategy.

P0094

Can machine learning predict which AAV capsids are viable after 7mer insertion?

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Directed evolution (DE) has been broadly used to improve adeno-associated viral vectors (AAV) for gene therapy. Using this technique, AAVs have been bioengineered to efficiently target specific tissues. DE for AAVs starts from a highly-diverse library with millions of different mutants which is screened by applying a selection pressure over multiple rounds, eventually converging towards a few 'evolved' variants. Clearly, if the initial library is not diverse enough, AAV variants with advantageous properties might be missed. One of the most utilized protocols for producing a high diversity capsid library consists in inserting a random 7mer at amino acid position 588 of the AAV2 capsid. Although this approach generates many different variants with interesting properties, some of the modified genomes do not code for viable capsids, resulting in a decrease of the initial library diversity, and therefore of the DE throughput. As a consequence, understanding whether a certain insertion is viable or detrimental can improve the overall efficiency of future DE screens. To address this challenge, we started from massive datasets previously collected and trained different machine learning models capable of predicting which sequences are viable and which are not. By leveraging these models, we aimed to pre-screen variants and enhance the initial library's quality, ensuring a higher likelihood of producing functional capsids.

The diversity of the variant distribution in the initial libraries allows us to create challenging train and test splits, thereby building robust models. These models can accurately distinguish between viable and non-viable sequences, offering a powerful tool to streamline the DE process.

P0095

CRISPRa system to improve lab-scale production of rAAV

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Recombinant-AAV (rAAV) is one of the most effective and safest vectors used in gene therapy, due to their non-pathogenicity, low immunogenicity and broad tropisms. Indeed, rAAV is approved by the FDA and EMA for many gene therapy protocols aimed to add the correct copy of a faulted gene in the cell target for the therapy.

The gold-standard packaging cells for lab-scale production of rAAV are the HEK293T, however the risk of leftover of SV40 large T antigen raises safety concerns. Since T antigen knock-out has no impact on the rAAV production, other genes must be critical.

We exploit the CRISPR/Cas9 Synergistic Activation Mediator (SAM) pooled human library to select genes whose modulation enhance the rAAV manufacturing in ReiCells, which are routinely used by ReiThera s.r.l. to package vectors for clinical trials. ReiCells were transduced with lentiviral vectors to generate cells expressing dead-Cas9, the transcriptional activator VP64, and two transcription co-activators, HSF1 and p65 (SAM-ReiCells). A gRNA library consisting of 70,290 gRNAs targeting 23,430 genes was cloned into a rAAV vector expressing the EGFP (AAVgRNA_EGFP). The complexity of the library after gRNAs cloning into AAV plasmid, and subsequently AAVgRNA_EGFP production, was tested by NGS (Bio-Fab research, Rome, Italy). The results showed that the 99.7% of the gRNAs were detectable indicating that in the AAVgRNA_EGFP library all the 23,430 genes are targeted by at least two gRNAs.

The AAVgRNA_EGFP library was serially amplified and rescued in SAM-ReiCells. The rAAV supernatant collected from the last round of the viral growth was used to PCR-amplify gRNAs which underwent a targeted NGS in order to rank the enriched gRNAs targeting genes whose upregulation benefit AAV production.

These genes will be overexpressed in ReiCells to generate a cell line with improved manufacturing capacity, which will be tested for the production of rAAV with clinical relevance. As relevant application, we are investigating the AAV CBE vectors tailored to a specific mutation, c.1A>G, in the exon 1 of *EMD* gene, which abolishes the start codon and cause Emery-Dreifuss Muscular Dystrophy Type 1 (EDMD1), a rare genetic X-linked disease due to the absence of the emerin protein. To restore the wild-type start codon, a dual-rAAV split-intein CBE system was designed. AAV CBE vectors will be produced using serotype DJ, 2, 8 and 9 and used to transduce EDMD1 myoblasts. AAV-treated EDMD1 myoblasts will be then evaluated by genomic analyses to detect the correction of the first codon and potential by-stander effects, as well as restoration of emerin expression by immunofluorescence. These data will provide evidences about the clinical relevance of an optimized lab-scale rAAV production.

P0096

Transfer of an AAV Production Platform from Bench-Scale to 50 L: Experiences, Challenges and Learnings

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Adeno-associated viral (AAV) vectors are promising delivery vehicles for gene therapies, but manufacturing challenges with low yields and poorly characterized products hamper supply. The Cell and Gene Therapy (CGT) Catapult has developed an AAV production platform using transient transfection of suspension-adapted HEK293F cells. The baseline process was optimized at small scale (15 mL to 2 L) before transfer to 50 L manufacturing scale in a GMP facility. However, consistency runs at 2 L (n=7) and 50 L (n=2) revealed challenges achieving comparable performance between scales. To improve scalability, USP development focussed on scalable and controlled formation of the transfection complex, while DSP development focussed on improving scalability of polishing chromatography. Critical process parameters for transfection conditions were assessed using a design of experiment approach. Then the optimised transfection condition was scale to 2 L where controlled mixing methods for complex formation and addition were studied with a focus on improving scalability. The resulting process improved viral titre while maintaining product quality in a scale-down model. Those changes were successfully applied at 50 L, where next steps are GMP manufacturing.

P0097

Qualification of an mRNA expression assay for GOI, stability insights, and process evolution detection

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Potency assays for clinical gene therapy products are multifaceted, requiring continuous efforts throughout preclinical and clinical phases. Gene therapy potency measurements face many unique challenges including transduction, transcription, translation, protein modification, cellular localization, and ultimately, protein function. A potency test, along with several other tests, assesses product conformance: release testing, stability programs and comparability studies when manufacturing changes are made. Potency tests are critical to measure rAAV product attributes such as quality, identity, purity, strength, and stability. Here, we report the establishment of a sensitive and specific in vitro cell-based assay to measure mRNA expression from our client's ophthalmic gene therapy vector, using multiplexed qPCR.

Measurement of mRNA expression after transduction with our AAV vector was achieved in three phases: cell transduction, cell harvesting/RNA isolation, and detection via gene-specific RT-qPCR. We successfully optimized this gene expression assay and qualified the assay for use as a functional potency assay. Qualification evaluated the assay's sensitivity, specificity, accuracy, precision, RT-qPCR linearity, and total assay linearity.

The abstract presents an overview of a study conducted over a period of time, focusing on the analysis of mRNA expression levels across various projects. Data collected encompassed assessments of stability, process comparability, and device compatibility. Through trending analysis, this research aimed to discern any alterations in mRNA expression patterns and derive meaningful conclusions

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P0098

Automated quantification of AAV capsids and genome content using capillary-based western blotting as an alternate route of empty/full ratio analysis

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1: Merck Millipore

Adeno-associated viruses (AAVs) have been pivotal for gene therapy applications due to their (i) minimal pathogenicity and (ii) the ability to sustain stable ectopic expression in both dividing and senescent cells. Specifically, AAV serotypes 2 and 5 are preferred for their targeted tropism and robust transduction capabilities, therefore crucial for a more effective delivery of therapeutic genes. To acquire a more guaranteed virus quality and efficacy a precise measurement of empty to full capsid ratios is vital since imbalances can significantly affect expected gene delivery.

In this study, we have employed an automated capillary-based Western blotting system to enhance protein and DNA analysis. This system can automate the conventional steps of protein separation and immunodetection as found in traditional Western blotting, thus improving the

accuracy and consistency in determining this critical ratio. In brief, the process is streamlined by a fully integrated system that manages sample and reagent loading, size-based protein separation, antibody addition and incubation, washing, and detection. This automation can markedly reduce user error and increase reproducibility, delivering results in up to three hours, with picogram-level sensitivity and quantitation akin to ELISA. The system's capabilities include both chemiluminescent and superior fluorescent detection, enabling comprehensive molecular weight characterization and precise protein quantification.

In this context, we were able to establish a robust workflow for AAV2 and AAV5 analytics, with a capsid detection ranging from 1.00 E+10 to 5.00 E+11 VP/mL as well as for DNA in a range of 5.00 E+09 to 2.00 E+11 gc/mL. Our preliminary findings align closely with those obtained from ddPCR/ELISA platforms and mass photometry analysis, but still suggesting potential areas of improvement in the area of capsid titration. Further investigation has indicated the changes in antibody concentrations and volumes have a minimum impact on LOQ. Moreover, our efforts to analyze capsid proteins and DNA, in a multiplex fashion, have indicated that changes on (i) antibody solution as well as (ii) denaturation time and temperature showed that DNA antibody diluent is more effective than the protein antibody diluent, which still does not accurately display DNA bands. In fact, AAV5 DNA proved to be quite stable at higher temperatures (i.e. 95°C) for about 1 minute, but degrades at longer incubation times (3-5 minutes). At the same time, the viral DNA remained stable at 75°C at all time points tested (1, 3 and 5 minutes). Despite these advancements, the clarity of capsid bands was not sufficient, thus highlighting the need for further optimization. As such, our line of work has focused on refining these methods, particularly through developing a more strict standard curve using viral proteins (VP1, 2, 3) instead of empty capsids for better capsid titration. Additionally, efforts to integrate DNA and capsid detection within a single capillary will lead to throughput advances, which could significantly improve the precision of AAV vector quantification overall and, thereby, augment their therapeutic potential in gene therapy protocols.

P0099

Flow cytometry (FC)-based approach for high-throughput detection of adenovirus infectious titer in gene therapy applications

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In the realm of gene therapy and viral vector production, accurately measuring the functional titer of Adenovirus, in crude and purified samples, holds paramount importance. Traditional cell-based assays, such as the plaque assay or fluorescent focus forming unit assay (PFU or FFU, respectively) are routinely impacted by time consumption and enhanced subjectivity. As an alternate route, we have recently adopted a flow cytometry (FC)-based approach that can offer advantages in this regard.

FC-based infectious titer measurement typically reduces overall assay time when compared to conventional methods, thus providing data within 24 hours in an electronic format which ensures superior traceability. FC signal is counted automatically, eliminating the challenge of subjective operator to operator interpretation in more traditional methods. Moreover, a higher and more representative cell population can be readily and precisely counted (thousands of cells per second). As such, this method yields more accurate readout since it detects signals from specific

cell population and can distinguish heterogenous subpopulation(s), unlike the traditional methods that depends on signals from cellular monolayers.

In our study, we have used HEK 293 suspension cell culture (aka AC2 cells), which were co-incubated with adenovirus 5 (Ad5) containing samples, and subsequently stained for Hexon5 protein using a FITC-labeled antibody. The resulting percentage of Hexon5 positive population was converted to FFU/mL according to a standard curve of known titer obtained with a positive control.

The future aspect for this method development involves exploring different cell lines for infectivity-based analyses, therefore assessing how adenoviruses may interacts and transduce distinct cell models and providing insights into tropism and efficacy. Also, adding a live/dead stain in the panel appears to enhance the assay's sensitivity and provide a more comprehensive understanding of viral behavior and any potential cytotoxicity.

By overcoming the constraints of traditional assays and providing superior accuracy, efficiency and traceability flow cytometry holds the promise of delivering a streamlined approach to measuring the functional titer of adenoviruses (and possibly others). Its capacity for high-throughput data generation renders it as a valuable tool, offering a time-saving solution for researchers in need of rapid and reliable results.

P0100

Comprehensive genomic profiling of Adeno-Associated Virus (AAV) vector preparations and precursor plasmids using nanopore sequencing

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Adeno-Associated Viral (AAV) vectors are typically small (~4.7 kb genome size), non-pathogenic viruses commonly used in gene therapy because of their relative safety, broad infectivity in dividing and non-dividing cells and stability, providing long-term, therapeutic expression. To date, there are six approved AAV-based gene therapies with several hundred in active clinical trials to treat a range of diseases. Due to rising use of AAV for gene therapy and recent changes in guidance, complete and accurate characterization is of paramount importance in drug development. Impurities or inaccuracies in the AAV preparation, including truncations of the vector genome, plasmid or cell line DNA contamination, or mutations in the transgene or inverted terminal repeat (ITR) regions can potentially impact safety and efficacy for downstream applications.

However, the production of recombinant AAV (rAAV) can naturally lead to the presence of truncated AAV genomes, host DNA, and DNA from both rep/cap and helper plasmids. These can potentially contaminate the final rAAV preparations. This highlights the importance of quality control (QC) in rAAV production before the preparations are used in downstream experiments. Currently, multiple methods including NGS, southern blots, qPCR and Sanger are required to assess rAAV integrity and purity. However, these methods are unable to accurately detect base-level transgene truncations, contaminants, inversions, or hotspots in ITRs.

Here, we demonstrate the ability to sequence full length (ITR-ITR) rAAV vectors and detect both contamination and truncated AAV genomes using a released end-to-end protocol that takes advantage of long, rapid, native nanopore sequencing. A quick library preparation turnaround time of less than five hours allows at least six AAV samples to be multiplexed, with approximately one hour sequencing time that enables results within a workday. Optimization of protocol steps including viral extraction and testing of annealing conditions were carried out to facilitate the accurate characterization of rAAV preparations. Additional to the released protocol, an analysis workflow on our tertiary analysis platform EPI2ME™ labs, called wf-aav-qc, facilitates fast and concise reporting for the easy assessment of contamination and truncation events, as well as the number of full-length AAV genomes and proportions of self-complementary AAV (scAAV) and single stranded AAV (ssAAV). Additionally, we have successfully sequenced full-length, primer-free plasmids used for creation of the rAAV for an additional level of quality control to ensure the constructs and vector backbones were error free.

In summary, we demonstrate rapid, comprehensive sequencing of native rAAV vectors from ITR-to-ITR, as well as the AAV precursor plasmids, identifying contaminates, discriminating between full-length and truncated genomes, and ITR mutations with optimized protocols for accurate characterization of AAV preparations suitable for GMP environments.

P0101

Characterization of recombinant adeno-associated virus 2 (rAAV2) capsid content variation for enhanced gene therapy efficacy

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Recombinant adeno-associated viruses (rAAV) have revolutionized the field of gene therapy, exhibiting immense potential in treating a diverse range of diseases. Understanding and characterizing the AAV capsid content variation is crucial for optimizing their efficacy. Here, we will present AAV2 capsid characterization data from upstream and downstream vector processing.

Our study aims to comprehensively characterize the critical quality attributes of AAV2, including empty/full ratios, partially filled capsids, and various biophysical properties. By leveraging advanced analytical techniques, we elucidate the impact of these factors on the process performance of AAV2 in gene therapy applications.

The investigation of AAV2 capsid content variation is vital for unraveling the complexities associated with vector production and optimizing therapeutic outcomes. The findings presented shed light on important considerations for the design and development of AAV-based gene therapies.

P0102

Efficient rAAV8 Capsid Quantification in Upstream Process Development with Two-Dimensional Liquid Chromatography

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High viral titer and a significant proportion of full capsids are essential in upstream processes for the successful creation of recombinant adeno-associated viruses (rAAV) in gene therapy applications. PCR methods can quantify the number of capsids containing the desired target in crude materials, but techniques precisely identify the ratio of empty to full rAAV, such as AUC and TEM require the purification and concentration of samples. Utilizing the combination of biochemical techniques such as (d)dPCR and ELISA is a prevalent strategy for assessing the ratio of empty to full components in raw samples, however, this method is time-consuming and cannot be used for real-time monitoring throughout the manufacturing process. In order to address these difficulties, the PATfix AAV Switcher, an automated analytical system with two columns, was developed. This paper presents a two-column analytical approach for accurately measuring the amount of empty and full capsids in crude rAAV8 samples, without the need for complex sample preparation. An analytical example will demonstrate the influence of various variables on the ratio of empty to full capsids during the production process of rAAV8. Furthermore, this poster will address the effect of contaminants, such as proteins found in the lysed samples upstream, on the detection limit and the minimum concentration of full capsids that may be accurately detected.

P0103

An optimized baculovirus generation process and novel ITR-transgene design for high quality AAV production using the insect cell production platform

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Development of a stable, robust and large scale AAV manufacturing process at low cost of goods is a major hurdle in the Gene Therapy field. The Baculovirus/insect cell production system has the potential to overcome these challenges but relies on efficient generation and scale up of intrinsically genetically unstable recombinant baculoviruses. Here, we developed a highly efficient recombinant baculovirus generation process with high rescue efficiency during plaque purification. Furthermore, we engineered a novel molecular ITR-Transgene design to improve baculovirus genetic stability. To this end, we used the Annogen SuRE™ technology to identify a small human promoter sequence with desirable sequence characteristics. When incorporated into a recombinant baculovirus, the VectorY ITR-Transgene design provides high genetic transgene stability whilst retaining high AAV production yields, essential for manufacturing scale up.

Furthermore, we utilized next-generation sequencing techniques to characterize the AAVs, showing high genome integrity and low contaminating DNA content. Lastly, we show that the AAV batches produced on this production system exhibit high transduction and transgene expression in human iPSC-derived CNS cell types and *in vivo* in mice and NHP. In conclusion, we present efficient production of stable recombinant baculovirus for high quality AAV production in insect cells.

P0104

Introduction to Genevoyager's One-Bac 4.0 System: Addressing the Bottlenecks and Challenges of Adeno-Associated Virus (AAV) Manufacturing

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Viral vectors play a crucial role in gene therapy, with Adeno-Associated Virus (AAV) emerging as the preferred vector for *in vivo* gene therapy due to its safety profile and the ability to sustain prolonged gene expressions. AAV offers advantages such as low immunogenicity, broad tropism, and a variety of serotypes.

Genevoyager's proprietary One-Bac 4.0 system marks a novel development in large-scale AAV production platforms, addressing the bottlenecks associated with commercial development of gene therapy products.

Key Features:

1. Optimize the Cap and Rep expression cassettes to increase the AAV full capsid ratio and enhance infectious activity
2. Achieve stability and yields ranging from 1E+15 to 5E+15 vg/L during scale-up manufacturing (500L-2,000L)
3. Ensure a safety profile with minimal impurities, and the absence of replication competent AAV (rcAAV)
4. Deliver high potency through increased infectivity and high full capsid ratios (＞70% in crude harvest), enhance the therapeutic efficacy
5. Provide high accessibility with reduced manufacturing costs, enabling an affordable pricing model that addresses the medical needs of a broad patient population

The innovative One-Bac 4.0 system effectively tackles the challenges in AAV manufacturing by offering high yields, sustained stability, elevated full capsid ratios, enhanced infectious activity, reduced cost, and accelerated production timelines. This system introduces a new approach for scalable and efficient production of AAV vectors.

P0105

Modulating Endocannabinoid Levels for Pain Control: CRISPR-Mediated Inactivation of Faah and Magl in Peripheral Nociceptive Neurons

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Targeting the enzymes fatty acid amide hydrolase (*FAAH*) and monoacylglycerol lipase (*MAGL*), which regulate endocannabinoid levels, offers a promising strategy for pain relief. To achieve loss of function of *Faah* and *Magl* in mouse cells, third-generation adenoviral vectors expressing CRISPR/Cas9 were designed with guides targeting the coding regions of these genes. Initial validation of the RNA guides by electroporation of the corresponding CRISPR/Cas9 RNPs in mouse embryonic fibroblasts (MEFs), demonstrated efficient generation of indels in the predicted regions consistent with loss-of-function mutations. RT-PCR analysis confirmed a significant decrease in *Faah* and *Magl* expression in electroporated cells. Assessment of endocannabinoid levels in treated cells by HPLC-MS analysis revealed changes in the endocannabinoid profile in accordance with the functional loss of each hydrolase. After construction and production of the CRISPR-carrying adenoviral vectors, their efficacy in inhibiting Faah and Magl expression was confirmed by MEF infection. In order to inactivate Faah and Magl in nociceptive neurons of dorsal ganglia, intrathecal injections of CRISPR adenoviral vector preparations were performed in mice. The initial assessment of paw withdrawal response in mice injected with adenoviral vectors, compared to sham-treated controls, was inconclusive when measured using an automated Von Frey anesthesiometer. Dorsal root ganglia dissections and individual genotyping of each ganglion were initiated four weeks post-injection. Sanger sequencing unambiguously revealed gene editing in a small fraction of genotyped ganglia, with varying editing levels observed between animals. Changes in the endocannabinoid lipid profile of tissue from dorsal, lumbar, and thoracic dorsal root ganglia from mice injected with adenoviral vectors for loss of function of Faah and Magl were consistent with decreased activity of each enzyme. This study demonstrates in vivo gene editing in dorsal root ganglion cells. It opens the door to using gene editing to adjust endocannabinoid levels in the peripheral nervous system as a potential pain treatment.

P0106

Biodistribution and safety of AAVrh10 and AAVrh74 viral vectors targeting Schwann cells in PNS tissues of Non-Human Primates

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Adeno-Associated Vectors (AAVs) proved to be a valuable tool for treating neurological disorders focusing on diseases of the Central Nervous System (CNS). Biodistribution and expression of AAVs and especially of AAV9 in the CNS of Non-Human Primates (NHPs) has been studied extensively, in contrast to the Peripheral Nervous System (PNS). Our aim was to examine the efficacy and safety of intrathecally injected AAVrh10 and AAVrh74 when targeting the PNS tissues for the treatment of inherited neuropathies. In particular, we aim to study the efficacy of both serotypes carrying a plasmid driven by the human Schwann-cell specific Myelin Protein Zero (hMPZ) promoter to transduce myelinating Schwann cells throughout the PNS of NHPs following lumbar intrathecal delivery.

In order to study the efficacy of AAVrh10 and AAVrh74 to target NHP Schwann cells, we delivered the vectors expressing either the *Egfp* or the *GJB1* gene under the control of the Schwann-cell specific promoter hMPZ by lumbar intrathecal injection. NHPs were divided in six groups: Groups 1 and 2 received the AAVrh10-*MPZ.EGFP* or AAVrh74-*MPZ.EGFP*, at the dose of 8.14E13 vector genomes (vg); Groups 3-6 received AAVrh10-*MPZ.GJB1* or AAVrh74-*MPZ.GJB1* each expressing the *GJB1* gene that is associated with X-linked Charcot-Marie-Tooth demyelinating neuropathy (CMT1X) at two different doses (3E13 and 8.14E13 vg), and finally Group 7 received the vehicle and served as the control group. NHPs were sacrificed 8 weeks after injection and PNS, CNS and peripheral tissues were collected. For peripheral nerve analysis, we studied the anterior lumbar roots, sciatic, femoral, branchial, optic and ulnar nerves. Vector genome copy numbers (VGCNs) were determined in PNS, CNS and peripheral tissues of all groups. Inflammation was studied in all tissues of all groups while expression analysis was performed only in PNS tissues of the EGFP injected animals.

In-life observations did not indicate any abnormalities caused by the vector delivery or the vector itself, and we did not observe any changes in peroneal, sural and radial nerve conduction velocities. We observed only elevated liver enzymes (AST and ALT) 25 days following vector delivery in both serotypes expressing EGFP but not in the other groups injected with therapeutic gene expressing vectors. Biodistribution analysis showed similar efficacy of both serotypes to transduce PNS tissues in both doses examined, although the high dose proved to be more efficient compared to the low dose. Expression analysis showed that EGFP was expressed mostly in Schwann cells in both serotypes, with quantification underway. Finally, we observed elevated ratios of macrophages and B-cells in the livers of the animals receiving the EGFP-expressing vectors, which is in accordance with the elevated liver enzymes in these groups.

This study provides for the first time data demonstrating successful PNS biodistribution and Schwann cell-targeted expression of AAVrh10 and AAVrh74 in NHPs following a single lumbar intrathecal injection at clinically relevant doses, and without any toxic effects. Overall, we provide evidence that a clinically translatable AAV-mediated gene therapy approach targeting Schwann cells could potentially treat demyelinating peripheral neuropathies including CMT1X.

KA and KAK contributed equally

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P0107

T cell-specific *in vivo* gene delivery with DART-AAVs targeted to CD8

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Vector systems mediating highly specific gene transfer selectively into a defined population of therapy-relevant cells is key for many *in vivo* gene therapy applications, including *in vivo* CAR-T cell generation. Adeno-associated vectors (AAVs) in combination with highly specific binders, such as designed ankyrin repeat proteins (DARPins) were previously demonstrated to mediate targeted *in vivo* gene delivery. Here we studied DARPin-targeted AAVs (DART-AAVs) displaying DARPins specific for human and murine CD8. Insertion of DARPins into the GH2/GH3 loop of the capsid protein 1 (VP1) of AAV2 or AAV6 resulted in high selectivity for CD8-positive T cells with unimpaired gene delivery activity as compared to unmodified serotypes. Remarkably, the capsid core structure determined at high resolution was unaltered after modification, with the DARPins protruding from the particle surface detectable by single-particle cryogenic electron microscopy. In complex primary cell mixtures, including donor blood or systemic injections into mice, the CD8-targeted AAVs were by far superior to unmodified AAV2 and AAV6 in terms of selectivity, target cell viability and gene transfer rates. Moreover, we investigated the receptor binding selectivity of our targeted AAV vectors showing binding to 88 ± 8 % (n = 2) of all CD8+ cells in healthy PBMC, as well as B-ALL patient samples, in which the high majority of cells were off-target tumor cells. Reporter gene expression was detectable on 85 ± 5 % (n = 4) out of all CD8+ cells. Remarkably, on-target selectivity determined as percentage of CD8+ among GFP+ cells reached 98.1 ± 0.8 % (n = 4) in this setting. *In vivo*, up to 80% of activated CD8+ T cells were hit upon a single vector injection into conditioned humanized or immunocompetent mice. While gene transfer rates decreased significantly under non-activated conditions, genomic modification selectively in CD8+ T cells was still detectable upon Cre delivery into indicator mice. In both mouse models, selectivity for CD8+ T cells was close to absolute with exceptional detargeting from liver. These data demonstrate that capsid insertion of DARPins mediates highly selective and efficient gene transfer into mouse and human T lymphocytes. Thus, the CD8-AAVs described here expand strategies for immunological research and *in vivo* gene therapy options substantially and facilitate translatability between animal preclinical studies and human applications.

P0108

Towards a novel, regulatable pharmaco-genetic treatment for Anti-Neutrophil Cytoplasmic Antibody (ANCA)-vasculitis using AAV vector encoding DNaseI

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Neutrophil extracellular traps (NETs) are formed by the release of DNA by neutrophils as a natural response to capture and kill microorganisms, but dysregulated NET formation also underlies numerous diseases, including autoimmunity, neurological conditions and cancer. DNaseI is a potential therapeutic as it degrades extracellular DNA in NET's leading to the removal of the cytocidal and pro-inflammatory proteins that decorate these structures. However, the clinical utility of DNaseI is limited by the short half-life of the enzyme (< 6 hours). In this study, we show twice daily intravenous (IV) injection of recombinant DNaseI (rDNaseI) is therapeutic in a mouse model of ANCA-vasculitis. The disease in humans is caused by autoantibody activation of neutrophils resulting in NET formation that causes severe damage to the small blood vessels of the kidney leading to glomerulonephritis. Current immunosuppressive treatments for ANCA-vasculitis patients prolongs life beyond a year, but with unwanted side effects including a 70% increase in infectious diseases along with additional toxicities and significant danger of relapse. To develop DNaseI as an alternative therapy, and to overcome the issue of short half-life of serum rDNaseI, we tested an AAV vector encoding murine DNaseI transgene (AAV-mDNaseI) in the ANCA-vasculitis model. AAV-mDNaseI induced up to a ~200-fold stable increase in serum enzyme activity levels over endogenous levels without apparent toxicity. The vector was superior to twice daily IV rDNaseI injection in the ANCA-vasculitis model because not only did it reduce kidney injury and inflammation similar to IV rDNaseI, but vector treatment also reduced albuminuria and MPO-ANCA, an outcome not previously achieved in this model with any other NET inhibitor. Encouraged by these data, we sought to construct a clinical AAV vector candidate where transgene expression is pharmacologically regulated to control autoimmune flares. A "molecular switch" (Xon, Montey et al, Nature, 2021) was integrated between the liver-specific promoter and the human DNaseI (hDNaseI) coding sequence to permit the induction of transgene expression via oral administration of a small molecule (Branaplam). Preliminary dose-ranging studies confirmed low basal serum DNaseI activity in the absence of Branaplam and a 90-to 120-fold increase in levels after male and female mice received three doses of Branaplam at 1.5-mg/kg or 48-mg/kg. Serum enzyme activity returned to baseline 3 and 5 weeks after drug washout, depending on dose. In concurrent studies (using an AAV vector without switch) we explored substitution of the hDNaseI transgene with a hyperactive variant of DNaseI (vDnaseI) to increase vector potency. Compared to the 130-fold increase in serum activity in AAV-hDNaseI vector-treated mice, AAV-vDNaseI induced a 470-fold increase in activity levels in male mice. However, only a 70-fold increase was observed in female mice. Differences across genders were reduced and activity levels enhanced by fusion of the vDNaseI with three modules of Carboxyl Terminal Peptide derived from human gonadotropin, which increases enzyme half-life, leading to ~1200-fold and 630-fold higher enzyme activity in male and female mice, respectively. Further studies are in progress to determine the therapeutic efficacy of pharmacologically regulated DNaseI expression in the ANCA-vasculitis model.

P0110

Characterizing the tissue specific regulation and epigenetic response to AAV9-SMN1 gene therapy for Spinal Muscular Atrophy (SMA)

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AAV-based gene are poised to have a large impact on rare diseases like Spinal Muscular Atrophy (SMA), a neurodegenerative disorder characterized by motoneurons loss and skeletal muscle atrophy due to the loss of *SMN1*. ZOLGENSMA, an AAV9-mediated gene therapy, was approved for SMA infants enabling significant milestones, including independent mobility, a decade after the injection. Despite this success, understanding how AAV-mediated gene therapies are regulated is crucial, particularly for genes requiring stable expression. An effective gene therapy must mimic the native regulation of the gene it replaces. However, little is known about how the AAV episome is regulated in different tissues overtime and its impact on long-term stability of the patient's epigenome. Using our gene therapy approach for SMA (AAV9-PGK-SMN1), delivered to SMA mice at postnatal day 1, we characterized the regulation of AAV9-delivered SMN at different biological levels in the spinal cord and the muscle and studied the host's epigenetic profile post-therapy. Animals were followed at days 3, 7 and 14 post-injection, key moments in disease progression. Additionally, to understand long term-regulation, we analysed animals at 6-months. We first characterized the diffusion of the viral genome to the different target tissues. We performed a viral genome copy number (VGCN) analysis and found equivalent fast and efficient transduction of the muscle and the spinal cord using AAV9-SMN1 gene therapy maintained on the long-term (6 months) without virus loss 14 days post-injection (P14). In wildtype mice, SMN mRNA expression is similar in both tissues, however, the SMN protein is 2-fold more expressed in the spinal cord, indicating protein-level regulation. The AAV9-mediated therapy restores this tissue-specific regulation despite the ubiquitous PGK promoter, leading to SMN (mRNA and protein) levels comparable to controls in the spinal cord but a decreased protein level in the muscle. Given this discrepancy in protein levels, we wanted to determine the response of muscle. We assess the restoration of the tissue using RNA-sequencing, and observed that at day 14, the transcriptome of the tibialis anterior muscle was indistinguishable from controls and was maintained at the long-term. This led us to hypothesize that despite the suboptimal rescue of SMN levels, the minimum threshold of SMN protein needed for functional effect was achieved. To test the gene therapy's effect on the epigenome, we profiled the 5-hydroxymethylcytosine (5hmC) landscape in muscle at P14 and 6 months. 5hmC marks gene expression sites, so combining 5hmC profiling with RNA-sequencing reveals active genes (5hmC + RNA) and predict gene that will be activated (5hmC only). At P14, most SMA-associated 5hmC changes were restored by AAV therapy, but the profile also showed new 5hmC changes not seen in WT or SMA mice, likely induced by AAV injection. By 6 months, these changes were not detected, confirming that AAV9-PGK-SMN1 gene therapy restores the 5hmC landscape in adult mice, despite transient changes at P14. We now aim to understand the parameters that control the efficiency of the AAV mediated gene therapy in this system and to use this as a base to rationally design new gene therapies.

P0112

Therapeutic modulation of the epigenome in female OTC-deficient patient hepatocytes with skewed X inactivation using Adeno-Associated Viral (AAV) vectors: Towards targeted X-chromosome reactivation

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Realization of the immense therapeutic potential of epigenetic editing requires development of clinically predictive model systems that faithfully recapitulate relevant aspects of the target disease pathophysiology. In female patients with ornithine transcarbamylase (OTC) deficiency, an X-linked condition, skewed inactivation of the X-chromosome carrying the wild-type OTC allele is associated with increased disease severity. The majority of affected female patients can be managed medically, but a proportion require liver transplantation. With rapid development of epigenetic editing technology, reactivation of silenced wild-type OTC alleles is becoming an increasingly plausible therapeutic approach. We have successfully established a novel chimeric mouse-human liver derived from explanted diseased livers of two female infants in the FRG mouse to develop novel epigenetic therapies. Our recently published findings confirm that the chimeric mouse–human liver model recapitulated the native state in each infant's liver. We utilised this model to explore CRISPR-based epigenetic targeting strategies in combination with efficient Adeno-Associated Virus gene delivery to reactivate the silenced functional OTC gene on the inactive X chromosome (Xi) *in vivo*. Our work is currently exploring multiple strategies including locus-specific manipulation utilising CRISPR-dCas9 in combination with or without X-chromosome wide editing by targeting a major effector of X-inactivation, XIST RNA. To facilitate access to the Xi, we successfully eliminated XIST RNA *in vivo* in patient hepatocytes utilising a CRISPR/saCas9 fragment deletion approach. RNAscope and qPCR results indicate a significant reduction in XIST RNA levels with up to 90% of hepatocytes showing depletion of XIST. Importantly, targeted allele specific X-reactivation assay using NGS showed a small but reproducible evidence of reactivation from the healthy OTC allele. This study marks the first *in vivo* editing of the XIST gene in primary human hepatocytes *in vivo* to therapeutically modulate the epigenome. We are currently using this novel system to test combinatorial epigenetic editing approaches to achieve robust targeted reactivation of the functional OTC gene on the Xi offering an intrinsic cure.

P0114

AAV's next top model: Influence of animal species on directed evolution of myotropic AAV capsids

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A hallmark of directed evolution of AAV capsids for gene therapy applications is their ex or in vivo selection from diverse libraries, which are typically composed of hundreds of different variants (in the case of pre-selected, barcoded capsids) or up to billions (in the case of e.g. shuffled capsids). Recently, though, it has become increasingly apparent that the type of selection model can significantly affect and bias the outcome, evidenced by capsids that do not translate from cultured cells to animals or humans, or that even perform differently between various strains from the same species, such as mice or monkeys.

Here, we have systematically investigated and confirmed this phenomenon, by analyzing and comparing the results of iterative or single-round AAV capsid screening ex vivo in cultured murine muscle cells, or in vivo in the musculature of mice or of monkeys.

To this end, we initially screened a shuffled capsid library composed of AAV serotypes 1, 6, 8 and 9 upon systemic delivery in mice or monkeys, with a focus on the striated musculature as our major target of interest in this study. In mice, we observed a gradual loss of AAV1, 6 and 8 together with a concurrent enrichment of AAV9, which eventually contributed to about 72% of all capsid sequences at the end of the selection. In striking contrast, AAV9 but also AAV8 were diminished in macaques, while AAV1 and 6 became enriched and ultimately made up roughly 57% of all capsid sequences.

Based on these data, we next prepared a barcoded pool of 54 capsids enriched in the previous study and including three clusters of similar sequences, i.e., the AAV1/6-derived clusters 1 and 2 as well as the AAV8-derived cluster 3. Moreover, we added the parental AAV1, 6, 8 and 9 as benchmarks, as well as the AAV9-based peptide display variant AAVMYO that exhibits a pronounced muscle tropism in rodents.

Also in this secondary screen, we noted substantial differences in the composition and phylogeny of the capsids that performed best in each of three selection systems, i.e., murine C2C12 myoblasts or intravenously injected NMRI mice or macaques. Most notably, AAV9 and AAVMYO outperformed all other variants on the RNA level in the mouse musculature, where they were enriched 7.1- or 30.7-fold over the input library, respectively. In contrast, AAV6 and two derivatives in cluster 2 with ~97% identity to AAV6 performed much better in the macaques, where they were ~5-fold enriched over the input library. The lead benchmark in mice, i.e., AAV9, was enriched 1.8-fold in the macaque model and thus 3.9-fold less efficiently than in the mouse model.

Collectively, this work enables us to draw two major conclusions, the first being that AAV6 might represent a better scaffold than the commonly used AAV9 for capsid evolution in the primate musculature. Secondly, we validate the concern that the outcome of AAV library screenings is critically affected by the selection model, underscoring the need for concerted efforts across academia and industry to find solutions to this problem.

P0115

Multi-Properties Optimization of Myotropic AAV Capsid Through Combinatory Multi-VR Library and Deep-Learning Models

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Current adeno-associated virus (AAV) gene therapy using nature-derived AAVs is limited by non-optimal tissue targeting. In the treatment of muscular diseases (MD), high doses are therefore often required, but can lead to severe adverse effects. Previously, we have rationally designed a myotropic capsid, namely LICA1, by computationally grafting the TGFβ3-derived αVβ6-binding motif into the VR-IV of a liver-detargeting capsid backbone, a hybrid of AAV9 and AAV9rh74. LICA1 showed great improvement in skeletal muscle transduction and strong liver-targeting across species, including non-human primate. Here, we aim to further improve the LICA1 capsid in multiple aspects, including productivity, skeletal muscle transduction, and antibody escape, by exploring the combinatory multi-VR mutations outside VR-IV. We created a barcoded capsid library starting from LICA1 backbone, which diversified simultaneously the VR-II and VR-VIII sequences. First, a comprehensive site saturation mutagenesis was performed, revealed the impact of all single amino-acid substitution in AAV productivity and AAV transduction at mRNA level in the *in vitro* human differentiated myotubes. The combination of single amino-acid substitution with improved productivity or transduction results in a marked improvement in the corresponding AAV property compared to either random or combination of worsen substitutions. In parallel, we found that proportion of improved variants in both productivity and transduction drops significantly when more than 4 mutations being introduced. Therefore, by limiting the number of mutations introduced and combining the selected substitution, we further significantly increased the proportion of improved variants compared to LICA1 in each AAV property. Furthermore, deep-learning-based classification models for all studied AAV properties were constructed thanks to the high diversification of generated AAV dataset and are currently used to design next-round library with combined VR-II+VR-VIII mutations and improvements in both AAV properties. In summary, these data demonstrate the potential of combinatory multi-VR mutations and potentially deep-learning-based models to enhance the LICA1 capsid in multiple aspects, offering promising advancements for more efficient and targeted AAV gene therapies for muscular diseases.

P0116

Developing a stable cell line for AAV production

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Adeno-Associated virus (AAV) vectors have emerged as highly promising tools for in-vivo gene therapy in clinical applications. However, the large-scale industrial bioproduction of AAV faces challenges in terms of efficiency and scalability, primarily due to its reliance on transient transfection of HEK293 cell lines. Efforts to develop more scalable systems, such as AAV producer cell lines endowed with inducible viral gene expression system to initiate viral vector production “on demand” have been hindered by the toxicity associated with the leaky expression

of viral genes. We previously designed the CASwitch, a novel inducible gene expression system with minimal leakiness. It combines the CRISPR-Cas endoribonuclease CasRx with the state-of-the-art Tet-On3G inducible gene system and can act as a switch for gene expression in response to doxycycline administration [https://doi.org/10.1038/s41467-024-47592-y]. Specifically, in the absence of doxycycline, the CasRx cleaves a short sequence (direct repeat - DR) placed in the 3'UTR of the target gene's mRNA thus leading to the loss of its polyA tail and subsequent degradation, thus eliminating leaky gene expression. In the presence of doxycycline, the tet-transcativator fully expresses the target gene while repressing the CasRx, thus enabling the target gene to be maximally expressed.

Here, we developed a stable AAV producer HEK293T cell line by using the CASwitch to regulate the inducible expression of Helper genes (E2A, E4, and VA RNAI) derived from Human Adenovirus 5 (HAdV-5). To this end, we first engineered a minimal Helper gene cassette with a doxycycline inducible promoter driving a minimal Helper gene cassette (pTRE3G-E2A[DBP]-IRES-E4[Orf6]). We then integrated this cassette, together with the VA RNAI and the CASwitch components in the genome of HEK203T cells by PiggyBack and Sleeping Beauty transposases to obtain an AAV producer cell line. Cell viability and growth analyses indicated a higher metabolic burden when the integrated cells were continuously induced with doxycycline compared to both uninduced cells, and to wild type cells grown in doxycyline, suggesting that constant expression of the helper genes for prolonged periods causes a decrease of cell viability.

We then proved the inducer cell line can efficiently be used to produce AAV vectors on demand. To this end, we first expanded cells in a cell stack with medium without doxycycline. Once the desired confluence was reached, only the Transgene and Packaging plasmids were transfected, as opposed to the traditional triple transfection method. We achieved a production efficiency of 80% compared to the triple transfection in the presence of doxycycline.

We demonstrated that integrating the CASwitch control network into a stable HEK293T cell line for AAV production significantly enhances scalability and production efficiency by minimizing the reliance on transient transfection. This stable system offers a more efficient and consistent approach for AAV vectors suitable for clinical applications.

P0117

Engineered AAV Capsids for Efficient Muscle Transduction in Non-human Primates

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Systematic administration of Adeno-associated virus (AAV) vectors containing therapeutic payloads can achieve widespread muscle transduction and represents a promising treatment for muscular disorders. Typically, very high doses of AAV are administrated intravenously due to its relative low muscle transduction efficiency. However, high-dose delivery of AAVs has been reported to cause significant adverse effects, particularly liver damage.

Using our AAVarta® platform, an AI-aided AAV capsid evolution discovery technology, we aimed to engineer AAV capsids with enhanced muscle transduction and reduced liver targeting. The screening process relies on viral mRNA recovery in the muscles by using a novel potent muscle-specific promoter. The top capsid candidates from a diverse library were selected through

AAVarta® enrichment ranking analysis and individually produced as recombinant AAVs with reporter genes for validation.

One variant AVT919 showed significant improvements in cardiac and skeletal muscles of mice over MyoAAV2A and MyoAAV4A, benchmark myotropic AAV capsids. In Cynomolgus macaque, intravenous injection of AVT919 demonstrated up to 35-fold improvement over AAV9 in the muscles at transgene mRNA level, while the transduction in the liver is comparable to AAV9. To reduce the transduction of AVT919 in the liver, two focused libraries containing mutations in different regions were generated. After screening in non-human primates (NHPs), several AVT919 variants showed complete liver de-targeting while maintaining efficient muscle transduction as indicated by NGS data.

Top candidates were selected and packaged with a reporter gene or a therapeutic payload for validation studies in NHP. Analysis of transgene expression and vector genome copies in the tissues demonstrated that several variants achieved efficient muscle transduction and liver de-targeting in NHPs. Additionally, these novel myotropic variants demonstrated superior performance in head-to-head comparisons, both in a DMD mouse model and in NHPs.

Thus, these engineered myotropic and liver de-targeted AAV capsids could serve as safe and efficient delivery tools for muscle-targeting gene therapies.

P0118

The engineered AAVpo1.A1 vector transduces efficiently murine and human skeletal muscle fibers with liver detargeting in a model of X-linked myotubular myopathy

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X-linked myotubular myopathy (XLMTM) is a severe neonatal disease that affects mainly the skeletal musculature leading to respiratory insufficiency and premature death. Several therapeutic approaches were previously evaluated in animal models of the disease, including a serotype 8 adeno-associated viral (AAV8)-based gene therapy. Since recent advances in AAV bioengineering led to the development of novel capsids with increased specificity and tissue tropism, in the present study we assessed the therapeutic efficacy of an AAVpo1.A1 vector that contains a small peptide insertion in a natural capsid of porcine origin. We administrated intravenously an AAVpo1.A1 vector that carries a MTM1 expression cassette at various doses in *Mtm1* deficient mice and analyzed the effect for a 3-month period. We performed vector biodistribution and transgene expression analyses from various tissues and organs, providing evidence of an efficient tropism for skeletal muscles and liver detargeting. At a single dose of $2x10^{13}$vg/kg, this vector prolonged survival and corrected muscle mass, histopathology and strength of mutant mice.

Importantly, we showed that this engineered vector had also the ability to transduce efficiently human muscle xenografts in immunodeficient mice. Altogether, these data indicate that AAVpo1.A1 could be a useful vector for gene therapy of muscle disorders with potential application in humans.

P0120

AAV-mediated overexpression of miR-146a as a gene therapy strategy to promote axon regeneration in the central nervous system

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Neurodegenerative disorders, including traumatic injuries to the central nervous system (CNS) and neurodegenerative diseases, are characterized by early axonal damage leading to permanent neurological deficits and cannot be repaired. Axonal degeneration, neuronal cell death and, principally, the failure of axon regrowth are largely responsible for those long-term deficits. Modulation of several genes is required for efficient neuroprotection and regeneration, thus, understanding the role of gene regulatory molecules, such as microRNAs may lead to a more efficient therapeutic strategy. Indeed, some studies have manipulated microRNAs in order to promote neuroprotection and regenerative axon growth. Despite these advances, microRNA biology in neurodegeneration and regeneration, especially in CNS injury models, remains poorly understood. Among the various miRNAs recently identified with roles in the CNS, miR-146a emerges as a promising target to promote axon regeneration. In the present study we tested whether forced expression, by viral vector-mediated gene transfer, of miR-46a could modulate neurite and axonal outgrowth and regeneration in the CNS. We used recombinant adeno-associated viral (rAAV) vectors to overexpress miR-146 (rAAV.miR-146) in primary cortical neurons. The rAAV.miR-146 vector also express the fluorescent protein EGFP as a reporter gene. As control we used a rAAV vector that express only EGFP (rAAV.CTRL). To evaluate neurite and axonal outgrowth and regeneration we used primary cortical neuron culture from embryonic Wistar rats. First, we showed that transduction of cortical neurons with rAAV.miR-146a increases neurite outgrowth. Then, by using the Sholl analysis we found that rAAV.miR-146a enhances neurite arborization complexity. To evaluate the regenerative effect of rAAV.miR-146a, first, we used a scratch assay to damage the neurons and found that rAAV.miR-146a promotes neurite regeneration. Finally, using microfluidic devices that isolate axons and allow a lesion specific in the axons, we showed that rAAV.miR-146a also enhances axonal regeneration after axotomy. Mechanistically, target prediction analysis identified different mRNA targets of miR-146a and we found that the mRNA of TRAF6 is downregulated by rAAV.miR-146a in cortical neurons. Our findings demonstrate that miR-146a can enhance neurite and axon regeneration, suggesting that this microRNA may be an interesting gene therapy target to promote regeneration in the CNS.

P0121

Searching the hairpin in the haystack: Tracing the impact of AAV-ITR mutations

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While modulating the Adeno-associated viral (AAV) capsid has become a standard technique for the derivation of next-generation AAV vectors, engineering of the ITR (inverted terminal repeat, i.e., replication and packaging *cis* element) sequences possesses equal and complementary potential to enhance vector productivity, safety and efficacy. Strikingly, however, the impact of the ITR sequence and structure on these parameters remains largely unexplored. To facilitate manipulation of the ITRs, we developed a pipeline for mutant AAV ITR screening, that ranges from a vector construct design allowing for easy modification of plasmid ITR sequences and a modified Sanger sequencing-based method for ITR integrity confirmation, to a barcode strategy to track the ITR variants during production and transduction. For this purpose, each ITR variant is associated with a specific DNA barcode in the 5‘ UTR of a transgene cassette on the virus genome. Nanopore sequencing-based examination of ITR integrity in vector genomes revealed a previously undescribed *trans*-acting ITR-repair mechanism in which dominant ITR variants encoded in different plasmids can serve as repair template. This ITR repair mechanism can be circumvented by producing the critical variants separately, permitting AAV vector generation with a large array of mutant ITRs that are stably maintained in the viral vector genome. Subsequent barcode interrogation by deep sequencing enables the determination of barcode distribution at high resolution, which, in turn, allows for a quantification of the impact of the ITR mutations not only on productivity but also on vector performance after administration both *in vitro* and *in vivo*. To determine the impact of the ITR variants upon transduction, barcode sequencing from RNA as well as after enrichment of episomal DNA reveals the impact of the ITR sequences on vector functionality and the ability to form episomes. This is complemented by a novel strategy to simultaneously determine the integration into the genome of the transduced cells as well as the barcode sequence, thereby resolving the impact of ITR variants on vector integration. Altogether, our pipeline for ITR variant generation and tracing upon transduction allows for a comprehensive understanding of the impact of ITR variants both *in vitro* and *in vivo*, which promises to ultimately benefit the creation and optimization of third-generation AAV vectors combining capsid and genome engineering.

P0122

Analysis of Genomic Configurations *in vivo* After rAAV Delivery

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Recombinant AAV (rAAV) vectors are one of the most widely used vectors in gene therapy. Despite the clinical successes of AAV gene therapy, there remains a limited understanding of post transduction AAV Biology and genomic arrangements, and how these may impact therapeutic outcome. AAV vectors may mediate long-term expression of therapeutic transgenes by existing as extranuclear episomes. AAV-ITRs are crucial cis-elements necessary, not only for packaging, gene transfer and gene expression, but also for driving genome recombination resulting in stable circular episomes upon transduction. Despite the pivotal role of ITRs in AAV persistence, a comprehensive understanding of the genome configuration of AAV episomes and how these episomes mediate long-term persistence remains a knowledge gap in the field. To this end, we performed third generation long read sequencing of episomes extracted from liver after AAV delivery. Here, we discuss the nature of ITR-based recombination events and uncover structural insights into AAV genome intracellular fate and episome persistence. In addition, we investigated epigenetic modifications to AAV genomes post-transduction. Active histone modifications (H3K27Ac, H3K4me3), inactive histone modifications (H3K9me3, and H3K27me3) on AAV genomes were characterized using Cut and Tag assay. While both types of modifications were detected across AAV genomes, there was limited detection of inactive, by not active, marks on the ITRs. Correlation of specific genome configurations, unique epigenetic modifications and functional outcomes are currently being explored. Further, identification of host proteins and key pathways that play a potential role in establishing persistence, and thereby durability of expression, are underway. In summary, these findings not only increase our understanding of vector biology but may also open new avenues for modulating AAV episome persistence, as a strategy to potentially improve the efficacy of AAV-based gene therapies.

P0123

Selective DNA delivery through a modular AAV platform utilising Affibody binding domains

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Adeno-associated viruses (AAVs) are increasingly used in gene therapy, but the serotypes typically found and used in therapies today display limited tissue selectivity. This leads to high doses required for effective treatment and increasing risk for side effects as well as increased drug production costs.

We present a modular AAV platform for selective cell receptor targeting of AAVs through Affibodies (Z). Affibodies are small independently folding protein domains, engineered by phage display to selectively bind cell surface receptors with high affinity. We explored genetic grafting of Affibodies to the AAV viral protein VP2 and selected three surface exposed positions for our study. The generic utility of this potential platform was assessed by measures of capsid integrity, productivity, packaging, infectivity, selectivity, and modularity.

For two of the three insertion sites, the Z-AAV variants bound their intended target protein of the Affibody. Similarly, selective and highly potent infection was seen for these Z-AAVs against cells displaying their target protein. No binding or infectivity was however detected for Affibodies grafted to the third position. Through cryo-EM, differences in AAV morphology was quantified by computational comparison between the AAV particles. The Z-AAV that had no detected binding to its Affibody target had similar morphology as the original AAV template, while the Z-AAVs with detected target binding had diverging morphology compared to the original AAV. Production levels and packaging were similar for the non-binding and non-infectious Z-AAV variant compared to original AAV, while the potent Z-AAVs showed approximately halved production levels and packaging.

The modularity of the Z-AAV platform was then demonstrated by swapping the grafted Affibody on one of the potent Z-AAVs to five other receptor-selective Affibodies. The five Z-AAV variants selectively infected cells displaying the targeted receptor protein. Lastly, a potential therapeutic use of the Z-AAV platform was then shown in a cancer killing assay, where cancer cells were selectively killed by Z-AAVs.

In conclusion, the Z-AAV platform can be used as a toolbox to redirect an AAV to certain cell receptors. As Affibodies can be generated towards generally any target of interest by protein engineering techniques, the Z-AAV platform is versatile and can be used to create more selective AAVs for gene therapy applications.

P0124

Therapeutic efficacy of MIDI dystrophin variants via split intein dual AAV approach

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Duchenne Muscular Dystrophy (DMD) is a yet incurable muscle disorder affecting children and causing premature death. DMD is caused by genetic mutations leading to the absence of Dystrophin, a protein essential to preserve muscle integrity during contraction. In the last few years, AAVs have proved to be a promising tool for DMD gene therapy. However, due to the limited packaging capacity of AAVs, only shorter versions of dystrophin (microdystrophin) can be accommodated. Several microdystrophin products, although functional in animal models, are currently being investigated in clinical trials with results far below expectations. One hypothesis is that microdystrophin lacks crucial structural and signaling domains that are required for a full correction. Therefore, there is an urgent need to understand the cause of the limited therapeutic efficacy of short dystrophin versions in patients and develop new strategies. Our approach consists of the generation of larger versions of dystrophin (MIDI dystrophin) including additional key domains as compared to microdystrophin, to understand their contribution to ensuring optimal performance. We harnessed the protein trans-splicing ability of the inteins to generate the different MIDI dystrophin constructs based on the use of two AAV vectors, each carrying a

portion of the protein. We selected two MIDI dystrophin versions based on the expression levels of the reconstituted protein upon systemic delivery of the dual vector in a DMD mouse model. Preliminary data reveals a 100% reconstitution efficiency and high therapeutic efficacy of MIDI dystrophins as compared to an equal amount of microdystrophin level. The approach is being further validated in vitro, using our newly generated DMD iPSC-derived engineered muscle platform to improve the translatability of the therapeutic outcomes to patients. These encouraging results pave the way to further investigate the basis of key domain combinations leading to a protein version closer to the physiological dystrophin that can assure higher functionality to treat human DMD pathology.

P0125

Ligand-modified rAAV6 vectors combined with nanoblades allow high level gene knock-in in hematopoietic stem and progenitor cells without compromising cell survival

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Nanoblades represent a new technology based on a modified murine leukemia virus, where the viral structure protein gag is fused to Cas9. These viral particles are loaded with the Cas9 protein complexed with the gRNA and devoid of any viral genome. We showed that nanoblades were remarkably efficient for entry into human T, B and HSPCs thanks to their surface co-pseudotyping with baboon retroviral and VSVG envelope glycoproteins. Incubation of hematopoietic stem and progenitor cells (HSPC) with rAAV6 vector containing two homologous arms to the Wiskott-Aldrich syndrome (WAS) locus flanking a GFP expression cassette together with nanoblades, resulted in 50 % of stable expression cassette knock-in into the WAS gene locus in HSPCs. However, high doses of rAAV6 induced HSPC cell death. Comparing rAAV6 with rAAV2 encoding the donor DNA, we demonstrated that at high doses, rAAV2 was much less toxic and gave transduction levels in HSPCs equivalent to rAAV6. To improve donor template delivery, rAAV2 and rAAV6 were chemically bio-conjugated with a mannose ligand, *via* the lysine or tyrosine amino-acid residues exposed at the capsid surface. Our results showed high level transduction of HSPCs with mannose coupled rAAV6 vectors accompanied by a remarkable lower toxicity compared to the unmodified rAAV6. Mannose bio-conjugated rAAV6 for DNA donor delivery combined with nanoblades allowed efficient gene knock-in and increased survival of HSPCs from 10% to 80% as compared to the unmodified rAAV6, even in the most immature $CD34^{+}CD38^{low}CD90^{+}$ hematopoietic stem cell population.

Summarizing, the coupling of mannose on rAAV6 surface maintained high level donor mediated gene knock-in when combined with nanoblades without inducing significant toxicity for the HSPCs, an important feature for clinical translation of HSPC-gene editing strategies.

P0126

AAVone: An All-in-One Single-Plasmid System for Efficient and Streamlined Production of High-Quality AAV Vectors

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Currently, the most common approach for manufacturing GMP grade adeno-associated viral (AAV) vectors involves transiently transfecting mammalian cells with three plasmids (tri-plasmid system) that carry the packaging components necessary for vector production. This method is versatile and relatively fast, but scaling up to meet the demands of clinical applications continues to be challenging. The requirement for all three plasmids to be transfected into a single cell, the high quantity of input plasmid DNA (pDNA) required, and the need for ratio optimization among the three plasmids limits AAV production efficiency, introduces variability between production batches, and increases both time and labor costs. Here, we developed an all-in-one single-plasmid AAV production system, called AAVone. In this system, the adenovirus helper genes (*E2A*, *E4orf6*, and *VA RNA*), AAV helper genes (*rep* and *cap*), and the AAV vector genome are consolidated into a single compact pAAVone plasmid (13 kb backbone plus AAV genome). An AAV vector can be efficiently generated by simply transfecting the corresponding pAAVone plasmid into host cells. The AAVone system has yielded remarkable results, achieving unpurified yields of over 1x10^15 vector genomes per liter (vg/L) in suspension-cultured HEK-293T cells for most AAV serotypes. This represents a two- to four-fold increase compared to the original tri-plasmid system. For AAV9, the AAVone system has achieved crude lysate titers as high as 3x10^15 vg/L in HEK-293T cells and 1.5x10^15 VG/L in HEK-293 cells. AAVone system not only has highly effective across different cell types, but also shows compatibility with different transfection reagents (PEI-Max, FactoVIR, AAV-Max et al.,). Remarkably, the AAVone system demonstrated the ability to generate AAV vectors with genome sizes ranging from 2.2 to 4.7 kb, while the sizes of the corresponding plasmids varied between 15.2 and 17.7 kb. Moreover, the AAVone system exhibits low batch-to-batch variation and eliminates the need for fine-tuning the ratios of the three pDNAs, simplifying the production process and easy to scale up from flask to bioreactor. In terms of vector quality, AAV vectors generated by the AAVone system and the tri-plasmid system display: 1) Similar capsid composition, genomic pattern, ITR integrity, and infectivity; 2) comparable or slightly higher levels of *E2*, *E4*, *rep*, and host cell DNA genomic contaminants; 3) A notable increase in *VA RNA* and *cap* genomic contaminants; 4) A substantial reduction in bacterial origin of replication and antibiotic resistance gene sequences; 5) A marked reduction in non-functional snap-back genomes. Replication-competent AAV (rcAAV) testing was negative in 1x10^9 vg of AAV vector produced with AAVone system. The total DNA impurities can be further improved by reducing the amount of pDNA used during production. Reducing the pDNA also leads to increased full-particle ratios up to 10% in crude samples, further enhancing packaging efficiency and cost-effectiveness. In summary, AAVone represents a straightforward, cost-effective, and highly consistent AAV production system to make high quality and less DNA impurities AAV vectors–making it particularly suitable for GMP-grade AAV production.

P0127

Non-Specific Transcriptional Activity of Backbone Elements in Plasmids Designed for Gene Therapy Manufacturing in HEK293

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The AAV2 P5 promoter is extensively used in HEK293-based recombinant adeno-associated virus (rAAV) production systems to transcribe the Rep78 and Rep68 proteins. This promoter can function either in its original position relative to the Rep gene or downstream of the capsid gene, with its activity suggested to extend through the plasmid backbone. However, the P5 promoter often leads to the unintended packaging of DNA contaminants into rAAV virions, originating from sequences upstream of the P5 promoter on the Rep-Cap plasmid.

To address this issue, we have designed new constructs where the P5 promoter downstream of the Cap gene is removed. Additionally, in other designs, we have eliminated the Rep binding element (RBE) and upstream transcription factor binding sites from the upstream P5 promoter, as well as the upstream backbone elements. Our studies showed that removing the downstream P5 did not affect Rep78 expression. However, removing both the downstream P5 promoter and the backbone elements upstream of the truncated P5 promoter abolished Rep78 expression, indicating that it is not the complete P5 promoter downstream of the Cap gene, but the residual backbone elements that drive Rep78 expression non-specifically. While traditionally considered residual in recombinant plasmids, our investigation reveals a previously overlooked phenomenon: the non-specific transcriptional activity of these bacterial elements within eukaryotic cell lines such as HEK293.

This revelation holds profound implications for optimizing plasmid designs and improving the safety of gene therapies, particularly in the context of manufacturing recombinant adeno-associated viruses (rAAV). When situated upstream of viral components, these backbone elements inadvertently direct the expression of viral elements, potentially triggering immunogenic responses in the cells and patients. Mitigating this risk demands a meticulous approach to identify and address non-specific transcriptional activity, as the inadvertent packaging of these elements between inverted terminal repeats (ITRs) or the mispackaging of plasmids may result in the expression of undesired components, including the backbone elements. This study underscores the importance of refining plasmid architecture to mitigate unintended transcriptional consequences to safeguard the efficacy and safety of gene therapies.

P0128

AAV(Lung)s: a class of lung tropism AAV capsids with improved transduction efficiency in both mouse and NHP models

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Developing a lung-specific AAV (Adeno-Associated Virus) capsid is a significant focus in gene therapy, particularly for treating lung-related diseases such as cystic fibrosis, chronic obstructive pulmonary disease, and genetic disorders affecting the lungs. Different AAV serotypes (AAV2, AAV5, AAV9) and engineering variants (AAV6.2FF, AAV2H22, AAV2.5T and AAV9.452sub.LUNG1) have been reported to transduce in lung cells, organoids, and tissues with different animal models. With our ATHENA I platform, we confirmed that AAV9 performs better than other AAV variants at RNA level in the lung of C57B6 and B6C3 mice after systematically injection. However, AAV9 demonstrates significant transduction in the heart, kidney, brain, and liver tissues as well, indicating that it is not a lung-specific capsid. Interestingly, while AAV9 exhibits high expression in the lung, it simultaneously shows a low DNA copy number in the same tissue. Following the results from the ATHENA I evaluation, we systematically designed a series of AAV variants, which we have named AAV(lung)s. Our preliminary findings consistently show that certain AAV(lung) vectors achieve an approximately 1000-fold (B6C3 mouse) and 500-fold (C57B6) increase in DNA accumulation in the lung compared to AAV9 in the mouse model. AAV(lung) targets lung alveolar type II (ATII) cells, not airway cells in mouse model. Further study revealed that AAV(lung) VR-IV and VR-VIII contribute to its lung tropism. Destroying either of them dramatically reduces both RNA and DNA levels in lung. In the NHP model, AAV(lung) vectors demonstrated lung specificity and exhibited about a 10-fold increase in DNA levels and a 2-fold increase in RNA levels compared with AAV9. To further increase the RNA expression of AAV(lung), we evolved this capsid using our ATHENA III DNA shuffling platform. Most of he resulting AAV(lung) variants maintains high level of DNA accumulation in lung in both mouse and NHP models. One of AAV(lung)s, AAV(lung)-19, showed ~25-fold RNA level in NHP lung compared with AAV9. Overall, we have developed a series of AAV capsids specific to mouse and NHP lungs, which will be beneficial for future lung gene therapy applications.

P0129

Identification of high potency and tissue specific promoters with massively parallel screening

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Promoters are an integral component to any effective gene therapy. Desired characteristics of promoters include safe and effective levels of transcriptional activity, short length, and tissue specificity. A potent promoter may allow for a therapeutic effect with a lower dosage, which could lower immune responses and manufacturing costs. A short promoter leaves more space for the transgene and mitigates the cargo capacity constraints of current gene therapy delivery methods. Shorter promoters are especially useful for central nervous system applications because neuronal genes tend to have a longer coding region compared to non-neuronal genes. Here, we developed a massively parallel reporter assay (MPRA) to screen a synthetic library of over 240,000 promoters that are 182 base pairs long. This flexible AAV-based platform can be applied to diverse model systems including primary human tissue, IPSC-derived organoids, and non-human primates. Initial screening in transfected mouse Neuro2A cells identified hundreds of potent promoter candidates, of which 34 were selected for independent validation using flow cytometry. We identified 15 promoters exhibiting folds higher expression than CAG despite a 10-fold reduction in size in Neuro2A cells. Furthermore, 5 of these promoters are stronger than CMV and 3-fold smaller. In the human Huh7 cell line, all 15 promoters have lower expression than CAG

indicating their specificity. In parallel, potent promoters were identified by this platform in human myotubes, primary mouse neurons, mouse liver and the mouse gastrocnemius muscle. Independent validation of single candidates confirms the strength of our candidate promoters *in vivo*. These selected promoters can then be further engineered using machine learning coupled with rational design to increase promoter potency. This approach allows screening of hundreds of thousands of rationally designed small promoters (<200bp) capable of driving strong transgene expression in complex model systems. Our selected promoters harbor great potential for future gene therapy applications.

P0130

Improved recombinant AAV vector production via molecular evolution of the viral Rep protein

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In the rapidly advancing field of gene therapy, recombinant Adeno-associated viruses (rAAVs) stand out as highly promising viral vectors due to their favorable safety profile, proven long-term expression levels, and broad cell and tissue tropism. The increasing number of clinical trials based on AAV vectors underscores the need for more efficient production processes to yield high vector titers and quality. A significant challenge in rAAV production is the efficient packaging of the genome into the viral capsid, with empty or partially filled capsids often constituting over 90% of the originally produced material.

To address this issue at the genetic level, we created a highly complex library of the rep open reading frame (ORF) using DNA family shuffling with the parental AAV serotypes (1-13). We then subjected this library to directed evolution in an AAV producer cell line to select for Rep variants that increase rAAV production. Following each selection round, single clones were isolated and analyzed. Strikingly, after the first round, enrichment of specific hybrid Rep domains was observed in 2 out of 10 clones. By the second round, a single hybrid Rep clone was notably enriched in 4 out of 12 clones. Subsequent comparisons of these selected clones for rAAV production revealed significant differences in their ability to package AAV2-based viral genomes into the AAV2 capsid. Remarkably, the hybrid Rep proteins demonstrated performance equal to or better than their parental counterparts, with observed increases in packaging efficiencies up to 2.5-fold.

These findings suggest that optimizing rep gene variants through directed evolution is a promising technique to decipher biological key functions and enhance rAAV production efficiency, offering a promising avenue for advancing gene therapy applications.

P0131

Exploration of Improvement Opportunities for Liver-directed Gene Therapy in Glycogen Storage Disease 1a (GSD1a) Through Protein Engineering and Sequence Optimisation

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Glycogen storage disease 1a (GSD1a) is a monogenic metabolic disease arising from the loss of functional glucose-6-phophatase protein (G6pase) expression in the liver. Lack of functional G6PC leads to impaired gluconeogenesis leaving patients at risk of severe hypoglycaemia under fasting conditions.

A successful gene therapy for GSD1a should offer widespread expression across liver hepatocytes as G6pase is an intracellular membrane protein. Current therapeutic efforts express wild-type G6Pase protein from a codon-optimised sequence driven by large portion of the endogenous promoter, placing the total transgene cassette outside the optimal transgene packaging size.

In our approach, we sought to promote robust and regulated G6Pase expression, while reducing packaging size to 4kb. In addition to full sequence optimisation for optimal codon use with removal of CpGs and splice signals, we also carried out protein engineering to increase stability, thus increasing expression of G6Pase. Finally, we benchmarked basal and insulin-regulated expression of our novel promoter design using the proximal G6Pase gene promoter (2.86kb).

Protein engineering approaches included homology and rational structure-based design. Compared to wild-type G6Pase, our novel protein variant showed a 2.5-fold expression improvement in liver cells. Screening of sequence-optimised variants yielded another 2.5-fold increase in expression compared to the natural human gene sequence. In addition, we also applied a bioinformatics approach to condense the endogenous promoter while retaining its activity. Candidate regulatory elements for G6PC were identified from epigenetic and transcriptional features in the wider G6PC genomic locus. These were combined with the core G6PC promoter to generate a condensed sequence of ~2.2kb. Our condensed promoter has similar basal expression to the large endogenous variant, and preliminary results indicate that it is sensitive to insulin treatment *in vitro*.

Taken together, our AAV cassette engineering has yielded the optimised elements required for broad G6Pase expression in the liver of GSD1a patients, driven by an endogenous promoter within a 4.0kb transgene cassette.

P0132

Novel AAV vectors engineered via directed evolution in human-relevant model systems outperform current benchmarks for clinical cardiac gene therapy

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Current gene therapy trials using recombinant adeno-associated viral (rAAV) vectors for treating hereditary and acquired heart failure (HF) often face the challenge of high clinical toxicity due to non-specific cardiac transduction. For instance, rAAV9-based gene therapy products are still widely used despite numerous cases of fatal liver failure in patients, primarily due to rAAV9's strong liver tropism. To address this, we developed the next-generation Cardiac AAV Platform (next-CAP) to engineer rAAV capsids with high cardiac transduction specificity, aiming for safer and more precise cardiac gene therapy medicinal products (GTMPs).

Using a combination of DNA-shuffling and peptide-display methods, we generated a highly diverse library from novel mammalian heart-derived AAV capsid sequences, comprising over 200 million variants. This library underwent iterative *in vivo* evolution across multiple species (mice, pigs, and non-human primates) through intravenous injections to identify AAV capsids with optimal cardiac transduction specificity and efficacy. Unique molecular identifiers, combined with long- and short-read sequencing of AAV genomes, allowed us to select a top-100 portfolio of cardiac-enriched AAV capsids, minimizing clinically relevant off-target effects on organs like the liver and nervous system.

Further testing involved co-injecting this top-100 vector portfolio alongside current clinical and pre-clinical benchmarks (rAAV9, rAAV2i8, rAAVrh74, rAAV2-THGTPAD, and myo-AAV) in mice, pigs, and non-human primates. This process identified a top-10 portfolio of novel heart-specific AAV capsids that demonstrated superior tissue-specificity compared to these benchmarks in the human-relevant farm pig and NHP model systems. Our new vectors showed an up-to 100-fold higher heart-to-liver vector genome copy ratio compared to benchmarks and optimal de-targeting of non-cardiac tissues such as the dorsal root ganglia, brain, and spleen in non-human primates. These capsids also displayed a consistent distribution across the left and right ventricles of pigs and non-human primates. Additionally, our workflow enriched HEK293 cell high-titer producing cardiac-specific rAAV capsids, facilitating scalable large-batch production necessary for addressing both rare and common cardiac diseases.

The next-CAP pipeline has thus enabled the creation of synthetic cardiac-specific rAAV capsids that significantly outperform current benchmark vectors in terms of transduction specificity and efficacy in human-sized pig and non-human primate hearts. With their good producibility, these vectors are ideally positioned for further evaluation of dose-response relationships and toxicity, moving us closer to clinical trials for therapeutic HF gene therapy.

P0133

Incorporation of transferrin receptor binder into AAV enables its efficient brain delivery for treatment of genetic CNS diseases

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We have established the world's very first technology that is designed to enable biologics to penetrate the blood-brain barrier (BBB). It is a clinically validated technology showing superior efficacy to that of conventional therapy in the central nervous system (CNS) when administered intravenously with an effector. The first drug utilizing the technology has proven its efficacy for CNS symptoms with excellent safety profiles in clinical trials and has been approved for marketing in Japan. Although this technology was initially developed for protein therapies, since the mechanism of action utilizes the transferrin receptor (TfR) mediated transcytosis, it can be applied to the adeno-associated virus (AAV) vector-mediated gene therapy approach when displayed on the surface of the AAV vector to gain its ability to penetrate the BBB. This is a big advantage in the gene therapy field, as conventional AAV vectors do not have enough efficiency to transduce cells in the CNS when administered systemically. Therefore, granting AAVs access to the CNS space will open doors to treat CNS diseases that are inaccessible by other existing approaches. Here, we successfully demonstrated that our uniquely designed TfR binder incorporated AAV vector efficiently transduced CNS cells with remarkable therapeutic effects in several human TfR knock-in mice models of genetic CNS diseases. Since the incorporated TfR binder binds both human and monkey TfR, efficient brain transduction in monkeys was confirmed as well, as expected. Unlike AAVs developed by a directed evolution approach, we know exactly why and how our AAV penetrates the BBB and that's the reason why we believe our vector serves as an extremely reliable tool for CNS gene therapy.

P0134

Development of a novel AAV variant for efficient in vitro and in vivo gene expression in rodent cardiomyocytes and engineered human heart tissue

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Adeno-associated viral (AAV) vectors are increasingly used for preclinical and clinical cardiac gene therapy approaches. However, gene transfer to cardiomyocytes poses a challenge due to

differences between AAV serotypes regarding expression efficiency in vitro and in vivo. For example, AAV9 vectors work well in rodents' heart muscle cells in vivo, but not in cultivated neonatal rat ventricular cardiomyocytes (NRVCM), necessitating the use of AAV6 vectors for in vitro studies. Therefore, we aimed to develop an AAV that could efficiently express genes in both NRVCMs and mammalian hearts. We used random AAV9 peptide libraries and selected variants on NRVCMs at the vector genome and RNA levels in parallel. The enriched library variants were characterized using high-throughput analysis of barcoded variants, followed by individual validation of the most promising candidates. Interestingly, we found striking differences in NRVCM transduction and gene expression patterns of the AAV capsid variants depending on the selection strategy. AAV variants selected based on the vector genome level enabled the highest transduction but were outperformed by AAVs selected on the RNA level regarding expression efficiency. Additionally, we identified a new AAV9 capsid variant that not only allowed significantly higher gene expression in NRVCMs compared to AAV6, but also enabled similar gene expression in the heart as AAV9 wild-type vectors after being intravenously injected into mice. Moreover, the novel variant enabled significantly higher gene expression in human engineered heart tissue (hEHT) than AAV9. Therefore, this AAV variant could streamline preclinical gene therapy studies of myocardial diseases by eliminating the need of using different AAVs for NRVCMs, hEHT, and mice.

P0135

Characterization of AAV Integrations in preclinical models of gene therapy using RAAVioli pipeline with long and short sequencing reads

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Adeno-Associated Viral vectors (AAVs) have been widely used in gene therapy to treat various genetic disorders. Although they are typically considered episomal vectors, several studies have shown that both fragmented and full-length AAV DNA can integrate into the genomes of host cells leading to hepatocellular carcinoma and clonal expansion in some preclinical models. However, methods and bioinformatic tools that provide a reliable and efficient assessment of AAV integration sites (IS) are required.

Here, we propose a sonication-based PCR approach combined with short-read sequencing and a bioinformatics pipeline called RAAVIoli (Recombinant Adeno-Associated Viral Integration analysis) to characterize AAV integration sites (IS) and vector rearrangements. RAAVioli utilizes Python and R scripts to parse alignments, identify IS, and reconstruct vector rearrangements using CIGAR strings. The robustness of our approach was demonstrated by characterizing AAV IS in a humanized liver mouse model, where human primary hepatocytes were transduced with a tomato-expressing AAV. In this model, vector insertions were previously characterized using an AAV-specific probe base selection method and long-read PacBio sequencing. Sequencing reads were analyzed in both cases using the same RAAVioli pipeline. A greater number of AAV IS were identified by PCR/short-read sequencing (N=730) as compared to the long-read sequence approach (N=370). AAV IS distributed similarly within the human genome showing the typical

preference of targeting CpG islands and transcriptional start sites. Moreover, 32 IS were shared among the two datasets, demonstrating the consistency of the results obtained independently from the sequencing platform adopted. Although the short-read sequencing method identified a lower number of IS (~10%) with rearranged AAV genomes compared to the long-read sequencing approach (~25%), it demonstrates significantly greater efficiency in retrieving AAV insertion sites.

Thanks to this platform, we recently characterized AAV IS in a liver-directed gene editing approach used to correct Wilson Disease (WD). WD is an autosomal recessive disorder caused by mutations in the ATP7B gene which is primarily expressed in hepatocytes and is involved in copper metabolism. Here, genome editing was accomplished by integrating a promoter-less human mini-ATP7B cDNA into the albumin locus (Alb-ATP7B) using a nuclease-free homology-directed repair (HDR) mechanism. The treatment corrected the disease phenotype in Atp7b-/- mice by promoting liver repopulation of edited hepatocytes. Analysis of AAV IS revealed that vector insertion did not only cluster near or within the albumin-edited site but also distributed throughout the entire gene, exhibiting a strand-orientation bias. Indeed, 80% of the Albumin IS have the integrated vector genome oriented in the same direction as the targeted gene. Interestingly, this phenomenon was not observed in Atp7b-/- mice injected with a control vector expressing GFP. These data suggest the occurrence of a selection mechanism favoring the survival of hepatocytes expressing the therapeutic transgene consequently to integration events that are not driven by HDR-mediated mechanism.

In summary, our work indicates that the development of reliable methods for the characterization of AAV integrations is fundamental to providing insights into the safety and efficacy of these vectors in several gene therapy applications.

P0136

Identification of AAV capsids for efficient lung transduction using a high-throughput screen in multiple pre-clinical lung models

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Vectors based on adeno-associated virus (AAV) have shown to be highly effective delivery vehicles for gene therapy applications in many organs and tissues. However, progress towards the development of gene therapies for genetic lung conditions has been impeded by the lack of AAV capsids that effectively transduce the human lung. While many AAVs have been identified or bioengineered for transduction of other tissues, most have not been examined beyond their target of interest. We hypothesised that some of these variants may have undiscovered lung tropism, as capsid properties that improve transduction in one tissue, could also improve the transduction of other tissues. This study aimed to perform an unbiased screen of a large set of currently available AAV capsids, most of which have not been developed to target the lung and have never been evaluated in a preclinical model(s) of human lung. Specifically, we performed high-throughput paralleled examination of 81 AAV variants for lung-tropism in multiple representative lung models.

Clinical translation of vector performance is highly reliant on the model used. Many AAV therapies that have shown promise in pre-clinical models have not had clinical impact. For this reason, we use a multi-model approach to assesses each of the 81 AAV capsids based on specific and unique functional characteristics. To this end, we utilised multiple cell models, including primary human airway basal cells (ABCs) as one of the screening models. ABCs are progenitors of the lung epithelial cell and undergo slow cell turnover, making them a desired clinical target for both gene augmentation and gene editing strategies, bringing the promise of long-term clinical benefit for many genetic conditions. However, we acknowledge the limitations of AAV transduction data obtained with cultured cells compared to the whole organ. Thus, we also employed human precision cut lung slice (hPCLS), allowing a unique opportunity to evaluate AAVs in primary human lung tissue explants as PCLS retain all structural cell types. Finally, we tested AAVs' ability to traffic to the lung following systemic administration in mice .

Our data shows a high degree of correlation in AAV capsid performance human cells and hPCLS; with AAV4/6.15.P01 (developed in human T-cells) transducing both the primary human ABCs and hPCLS with the greatest efficiency. While AAV4 was the best performer in mouse lung *in vivo*, AAV-KP3 (developed in human islet cells) and AAV-DJ (developed in a human hepatoma cell line) both displayed a consistently high degree of transduction efficiency across human and mouse models, demonstrating their ability to not only transduce the human cells of interest but also traffic to the lungs *in vivo*.

The study provided us with important insights into AAV capsid's structural determinants of lung transduction and the identified lung-tropic capsids are a useful starting point for pre-clinical experiments as well as potential strong candidates for clinical translation.

P0137

Developing oligodendrocyte targeted AAV capsids

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Gene therapy for oligodendrocytes holds promising potential for treating a wide range of demyelinating disorders such as multiple sclerosis and leukodystrophies. However, efficient and targeted delivery of therapeutic genes to these specialized glial cells remains a significant challenge due to the complexity of the central nervous system and the need for precision in targeting specific cell types. rAAVen Therapeutics has developed an innovative solution with the rAAptr (rational AAV peptide research) platform, which utilizes the rational design of adeno-associated virus (AAV) capsids to create tailored gene delivery vectors for cell-specific applications.

The rAAptr platform employs a rationally designed library of peptide inserts within the AAV capsid, generating a vast array of AAV variants. This approach enables the identification of highly efficient AAV variants in a single round of screening, significantly reducing the number of animals required compared to traditional directed evolution methods. These variants are subsequently screened for cell type-specific transduction by sequencing expressed DNA barcodes from the viral transgene, enabling the identification of AAV capsids that are optimally suited for targeting specific cell types within the brain.

In this study, we applied the rAAptr platform to develop AAV capsids specifically targeting oligodendrocytes. By designing a library enriched for oligodendrocyte-targeting peptides, we observed a significant shift in transduction profiles, favoring the white matter regions of the brain

where oligodendrocytes are predominantly located. Additionally, we identified capsid variants capable of dispersing from the ventricular system to adjacent brain areas, which is crucial for widespread gene delivery within the central nervous system.

To pinpoint variants with a high affinity for oligodendrocytes, we utilized fluorescence-activated cell sorting (FACS) to separate oligodendrocytes from non-oligodendrocytes, followed by sequencing of the viral barcodes. With this approach we could identify and validate AAV capsids with robust oligodendrocyte specificity. Among the promising candidates, a variant named AAV-Kingfisher10 demonstrated exceptional targeting efficiency. AAV-Kingfisher10 not only showed a high degree of specificity for oligodendrocytes but also exhibited the ability to effectively transduce cells in the white matter regions of the brain.

Furthermore, by cross-referencing these variants with those detected in brain tissue following intraventricular injection, we confirmed the presence of capsids that can both disseminate from the ventricle and preferentially infect oligodendrocytes. This dual capability is particularly important for ensuring that therapeutic genes can reach and effectively treat widespread areas affected by demyelinating disorders.

Our findings underscore the efficacy of the rAAptr platform in designing AAV vectors with precise cell type specificity. The identification of AAV-Kingfisher10 and similar variants represents a significant advancement in the development of more effective and targeted gene therapies for oligodendrocyte-associated disorders. This research not only provides a pathway for treating demyelinating diseases but also highlights the potential of rational design approaches in creating next-generation gene therapy vectors tailored for specific therapeutic needs, all while minimizing the use of animal models.

P0138

S/MAR-Containing AAV Vectors Result in an Increase in AAV Episomes and a Reduction in AAV Integration Sites in a Mouse Model With a High Rate of Hepatocyte Proliferation

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Scaffold/matrix attachment regions (S/MARs) are highly conserved ubiquitous DNA sequences that are involved in chromatin organization and gene regulation. Studies have shown that these sequences are able to increase expression and facilitate nuclear retention of exogenous DNA that contain them, allowing the exogenous DNA to replicate in synchrony with the host genome.

Recombinant adeno associated viruses (rAAVs) are vectors containing single stranded DNA that are broadly used for gene therapy (GT) due to their good safety profile and therapeutic efficacy. AAVs have a very low integration rate, although some integration events have been observed for both wtAAVs and rAAVs. Due to the non-integrative nature of AAVs, their episomal genomes are

lost upon division of the transduced cells, and thus therapeutic efficacy in growing organs, such as the infant liver, may be transient.

Hereditary tyrosinemia type I (HT1) is an autosomal recessive disease arising from mutations in the fumarylacetoacetate hydrolase (FAH) coding gene resulting in disruption of the tyrosine catabolism pathway. This leads to the accumulation of toxic metabolites that cause liver failure as well as kidney damage. The standard of care consists of daily dosing of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC) that prevents the production of toxic metabolites and can avoid the need for liver transplant.

Using an HT1 mouse model (Fah1R Tyrc/RJ), in which FAH-corrected hepatocytes have a selective advantage, we questioned whether the presence of a S/MAR sequence in an AAV expressing hFAH would favour maintenance of the AAV genome as an episome while inducing its replication in synchrony with hepatocyte regeneration, leading to a sustained therapeutic effect in damaged livers. Mice were injected with a subtherapeutic dose (1E11 vg/kg) of either a control AAV (AAV-FAH) or an AAV containing a S/MAR sequence. This sequence was placed in different configurations within the AAV vector to test its functionality and effect on hFAH expression. NTBC was withdrawn 2 weeks post AAV injection (p.i.) and body weight (BW) and serum biomarkers (SB), were followed periodically until 20% BW loss or to 70 days p.i., at which point the mice were sacrificed. Initial AAV transduction, viral genomes and hFAH expression levels in liver were quantified by qPCR. FAH^+ liver area was detected by IHC, and viral integration was analysed by S-EPTS/LM-PCR and TES.

Results showed that the S/MAR-containing AAVs achieved higher therapeutic effect than the control vector. Three different phenotypes were observed upon NTBC withdrawal based on BW and SB: failed, recovered, and stable. Survivors showed BW and SB normalization after approximately 50 days p.i. and the number of viral genomes and hFAH expression levels were similar across experimental groups. IHC showed FAH expression in clusters of positive hepatocytes in recovered mice, suggesting potential AAV integration and clonal expansion. Integration studies generally revealed higher presence of episomal AAV genomes, read as vector-vector junctions, in the groups treated with S/MAR containing vectors. These results indicate that not only the presence of the S/MAR sequence, but also its position within the therapeutic vector, influence the fate of the viral genome in the transduced cell.

P0139

Mirroring AAV gene therapy for haemophilia B: insights from an in vitro stably transduced humanised liver cell model

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Liver-directed gene therapy using adeno-associated viral vectors (AAV) shows great potential for treating monogenic disorders, yet the AAV life cycle in humans remains poorly understood. Here, we present an in vitro model using human hepatocytes stably expressing human factor IX (hFIX) after transduction with scAAV2/8-LP1-hFIXco, the same vector used in the St Jude/UCL AGT4HB clinical trial for haemophilia B (NCT00979238).

scAAV2/8-LP1-hFIXco was manufactured in HEK293T cells via triple transfection with a helper plasmid (HGTI), capsid plasmid (p2-8), and vector plasmid containing a codon-optimized human

FIX cDNA under the control of the liver specific promoter LP1. Huh7 cells, maintained with vitamin K-supplemented media, were transduced bi-weekly with scAAV2/8-LP1-hFIXco at a multiplicity of infection of $5x10^5$ for eight times (round 1) or nineteen times (round 2). Limiting dilution was used to isolate and expand monoclonal cell populations stably expressing hFIX. Vector copy numbers were quantified using digital PCR (dPCR). mRNA expression was assessed using RT-qPCR with transgene specific primers. hFIX antigen levels were quantified using an in-house validated ELISA protocol. DNA and RNA in situ hybridization (FISH) was assessed using RNAscope technology (Advanced Cell Diagnostics). Southern blot analysis was used to determine transgene configuration in these stable clones

Four clones [CL1, CL2, CL3(round 1), CL19 (round 2)] exhibited either high (CL1 & CL19) or low (CL2 & CL3) hFIX expression at steady state levels of > 0.2 µg/ml in the high clones or <0.08 µg/ml in the low clones, which remained stable over a period of 7 months. Digital PCR showed hFIXco DNA signal with significant inter-clonal differences ranging from 2.46 to 7.65 copies/cell ($p<0.0001$). RT-qPCR revealed varying mRNA expression levels across clones ($p<0.0001$), which correlated closely with protein levels. FISH showed a scattered nuclear hFIXco AAV DNA signal in high-expressing clones (CL1 and CL19) versus a focused single signal in low-expressing clones (CL2 and CL3), with a larger proportion of cells bearing the viral genome without hFIXco mRNA expression. Manual quantification of randomly selected fields from FISH images showed that Clone 1 had 144 DNA-positive cells out of 241, with 128 of these also being RNA-positive; Clone 2 had 90 DNA-positive cells out of 100, with 49 of these also being RNA-positive; Clone 3 had 170 DNA-positive cells out of 372, with 102 of these also being RNA-positive; and Clone 19 had 104 DNA-positive cells out of 104, with 98 of these also being RNA-positive. Concatemeric AAV vector forms were found in three clones (CL1, CL3, CL19) analysed by Southern blotting. Pre-digestion of genomic DNA with Plasmid Safe DNase suggested that most of the hFIXco pro-viral DNA was integrated in these serially passaged clones but a minority of vector genomes persisted as episomal forms.

This stable human in vitro model of liver transduction exhibits variability in AAV transgene transcription and translation, mirroring observations from human liver biopsy studies following gene therapy. These clones offer a valuable opportunity to study and understand the AAV life cycle in a context that closely resembles in vivo gene transfer to the human liver.

P0140

Bioconjugated AAV vectors with enhanced functionality and tropism

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Enhancing AAV functionality through capsid engineering has become an area of enormous growth for gene therapy. The most common method to achieve this is to use genetic engineering, whereby sequences encoding short peptides are inserted into exposed loops of the capsid proteins. While many new AAV genetic variants have been described with potential for clinical applications, this approach brings certain shortcomings, most notably the necessity to operate within the constraints of preserving the capsid integrity and vector yield, and the lack of knowledge of the underlying biological mechanism that endowed the selected new capsid variant with the new desired tropism.

Here we describe an alternative approach to capsid engineering based upon bioconjugation of targeting ligands onto exposed residues of an AAV capsid using biorthogonal chemistry. By implementing a post-manufacturing functionalization step, we can make virtually any AAV vector amenable to accept a ligand that will re-direct, modify or enhance its tropism. We show that our approach can accommodate multiple classes of ligands, from active peptides to full-length protein domains, allowing pre-determined human validated targets to be used as binding receptors, and resulting in enhanced AAV vector potency with no impact on yield. Our approach does not require extensive screening of capsids or targeting ligands, since it works from knowledge of the receptor-ligand expression profile and function to the desired tissue tropism. Depending on the ligand chosen we can add new functionalities to AAV, such as improving transduction across a variety of cell types, achieving cell-specific targeting, or increasing transport across the CNS.

As a further development of our technology, we have designed vectors that can be modified at specific sites thanks to the insertion of small amino-acid sequences in the capsid that can be enzymatically modified and used as an acceptor of ligand chemical conjugation, achieving the same cell-specificity and tropism enhancement that we obtained with our original approach.

Collectively, these data demonstrate that chemical modification of the AAV capsid can produce vectors with novel properties, circumventing limitations associated with genetic engineering. By applying optimized chemistry to clinically relevant AAV capsids, performance and specificity of AAV mediated gene therapy can be enhanced across highly coveted target tissues.

P0141

Key process improvements required when building a cost-efficient AAV manufacturing platform

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The development of an adeno-associated virus (AAV) manufacturing platform that balances cost-efficiency with high quality is crucial for advancing gene therapy applications. The pressure to reduce costs and increase yields has significantly influenced process development strategies, supported by the numerous manufacturing platforms developed in recent years. This study reviews the impact of upstream and downstream process improvements on reducing the cost per dose for AAV-based gene therapy. Beginning with the selection of starting materials, we demonstrated that careful screening of cell lines and transfection reagents is required to decrease process costs. We also determined how cell lines impact quality attributes such as the percentage of full and empty capsids and the level of host cell impurities, which have drastic consequences on downstream operations. By meticulously optimizing feeding strategies and transfection parameters, including plasmid ratios, we managed to decrease the cost of goods by a minimum factor of five. Since process performance is the primary driver of cost reduction, we then improved our downstream processes to deliver optimal product yields and purity. We used design of experiment methodology to screen starting materials and optimize our endonuclease treatment, chromatography, and filtration steps, achieving overall recoveries of 30-40% after polishing and enrichment of up to 90% full particles as determined by AEX-HPLC. In addition to development impacts, we assessed the effect of scale on manufacturing costs per dose, showing overall cost reductions for larger-scale manufacturing for high-dose products. Larger batch operations demonstrated greater cost savings compared to multiple smaller batches. Therefore, the scalability of the manufacturing platform is key to ensuring cost-effectiveness at commercial

scales. In conclusion, we have shown that analysing cost drivers is a valuable tool for prioritizing focus areas in process improvement during process development. This is particularly beneficial for processes producing viral vectors for gene therapies such as AAV, given the limited practical commercial experience and short development timelines. The findings from this work highlight the importance of understanding the relative costs of each process component.

P0142

Modular assembly of capsids allows generation of AAV variants passing the blood-brain-barrier in mice

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There are several known procedures to diversify the *cap* gene in Adeno-Associated Virus (AAV) vectors that may lead to improved transduction of target cells. The blood-brain-barrier (BBB) has been found to significantly hinder efficient gene transfer into the central nervous system (CNS). We used modular assembly to design and synthesize a barcoded plasmid library of 10^4 different capsid variants, which were based on the recombination of *cap* fragments from ten naturally occurring serotypes. Subsequently, a large-scale production of replication-competent AAV was performed in Pro10 suspension cells. Nanopore long-read sequencing confirmed that 98% of library diversity was retained after AAV production. Using the same data set, we were able to generate a look-up table for assignment of barcodes to capsid variants allowing high-throughput barcode Illumina sequencing for later variant identification *in vivo*.

Our AAV library was injected intravenously into C57BL/6 B6N mice (N=6) to track the specificity and efficiency of variants *in vivo*. Genomic DNA samples isolated from two punctures of 12 different tissues, including brain, and viral input were applied to polymerase chain reaction (PCR) for amplification of barcode sequences followed by Illumina sequencing. Subsequent bioinformatic analyses enabled the identification of 272 - 460 capsid variants per animal (read count >20) reaching the brain of which 156 were detected in all six mice. Enrichment of these variants was found to be significantly up compared to viral input and further investigation revealed patterns of certain serotypes in distinct positions of the assembled capsids favoring entry into the brain. Ongoing analyses may provide additional insights into capsid specificity across the remaining tissues.

The capacity of the library capsids to escape human neutralizing and binding antibodies may also be assessed by using intravenous immunoglobulin (IVIG), a pool of plasma antibodies from many different human donors.

In summary, we have demonstrated that our capsid design approach using modular assembly may allow us to generate AAV variants which can pass the blood-brain-barrier in mice supporting the improvement of CNS gene therapy efficiency.

P0144

In Vitro Binding to Human and NHP Orthologs of Candidate Receptors Identify Novel Systemically-Delivered AAVs with Enhanced CNS Tropism

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The prospects for using adeno-associated viruses (AAVs) to treat central nervous system (CNS) diseases have been strengthened by the discovery of capsids with peptide insertions that enhance CNS tropism beyond that of naturally occurring AAVs. However, the therapeutic utility of these engineered capsids has been complicated by the lack of cross-species tropism. To address this limitation early in the discovery pipeline, we have used an in vitro binding platform to select novel capsids that bind both human and non-human primate (NHP) orthologs of 10 receptors. Transferrin receptor (TFRC), CA4, ALPL and CD98 were assayed due to their known ability to transcytose AAVs in animal models, while the others in our list are highly expressed GPI-anchored proteins in human brain endothelial cells. The extracellular region of each receptor was expressed as a fusion protein and immobilized on beads to affinity purify capsids from libraries of engineered AAV9 with peptide insertions. The peptide insertions were either random or designed to uniformly sample the vast sequence space. These screens identified diverse sets of AAV variants that were reproducible, unique to each receptor, and featured peptide sequence motifs that matched published data or were novel. Many of the candidate receptor ortholog pairs (including ALPL and CD98) showed overlapping sets of binding capsids, while some (including TFRC and CA4) did not. An expanded capsid library was generated via diversification of the initial screen hits using a deep learning-based modeling and sequence generation framework, and this new library was tested in cell-based assays and animal models. Experiments using human receptor knock-in mice and NHPs revealed multiple novel capsids that utilize different receptors with improved CNS tropism compared to AAV9 when delivered systemically. Current efforts to further optimize CNS tropism will be reviewed.

P0145

Bioorthogonal chemistry for rAAV engineering: a versatile strategy to label the capsid with small molecules and proteins

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Recombinant adeno-associated viruses (rAAV) are currently extensively investigated as vectors for gene therapy. However, their use is still limited by both an important tropism (resulting in the difficult targeting of the cells of interest) and a compromised efficiency (due to their potential neutralization by the immune system). In the purpose of optimizing the rAAV vector and overcome these limitations, our group has developed expertise in the direct chemical bioconjugation of lysine or tyrosine residues on their capsid to allow covalent linkage of ligands with targeting properties. In particular, coating the rAAV capsid with carbohydrates such as *N*-acetylgalactosamine and mannose resulted in considerably improved gene delivery to the targeted cells in both *in vitro* and *in vivo* transduction studies. Despite promising results, it is now of particular relevance to be able to introduce easily and efficiently a broad range of functionalities to exploit rAAV-conjugates as vectors in a broad spectrum of pathologies. Particularly, introducing peptides and proteins with strong and highly specific targeting properties would represent an interesting tool to improve cell specificity. To that end, this study consists in the evaluation and optimization of a strategy that combines bioconjugation with bioorthogonal chemistry to access new rAAV-conjugates. First, rAAV2 was labeled with azido groups on lysines or tyrosines using previously developed bioconjugation strategies. Different levels of modification were obtained, according to biochemical assays and mass spectrometry. Then, we demonstrated the possibility to post-functionalize those vectors using optimized strain-promoted azide-alkyne cycloaddition (SPAAC) conditions. Vectors labelled with fluorophores, biotin or carbohydrates could be efficiently obtained when the azido-rAAV were reacted with the corresponding DBCO derivatives. More interestingly, the bioorthogonal click reaction was extended to conjugate rAAV2 with two relevant nanobodies, targeting specifically the immune receptors CD62L or CD45. The resulting functionalized vectors conserved an interesting transduction efficiency in HeLaT cells, while the modification seems to impact positively their transduction profile in immune cells lines in vitro even in presence of the HSPG-binding inhibitor heparin. With this strategy, we expect to open the chemical engineering of rAAV vectors to more complex and specific targeting structures such as proteins, and contribute in the optimisation of gene therapy treatments.

P0146

Bioconjugated adeno-associated virus-derived vectors: an alternative to enhance gene expression in targeted tissues

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In this study, we used innovative chemical bioconjugation techniques to enhance the efficiency of recombinant Adeno-associated virus (rAAV) vectors, named BioAAV, by selectively modifying lysine) or tyrosine residues on rAAV2 capsids with carbohydrate ligands. Combining chemistry to vectorology is an alternative to address essential challenges to fully harness the potential of these vectors. This was done through covalent coupling reactions involving nucleophilic addition of lysine amino groups with mannose isothiocyanate ligands, or aromatic electrophilic substitution of tyrosine phenol groups with mannose diazonium salt ligands.

Following thorough validation of the chemical coupling using a battery of analytical assays, we assessed the *in vivo* efficacy of these BioAAV2 vectors, each carrying an Enhanced Green Fluorescent Protein (eGFP) reporter gene expression cassette under a ubiquitous promoter, in retina and brain of rodent models. Our investigations showed that, following both subretinal injection and intrastriatal injection, BioAAV2 greatly enhanced vector transduction efficiency and gene expression. In mouse and rat retina, eGFP expression was one-log higher after the injection of BioAAV2 than after the injection of unmodified rAAV2. In mouse brain, expanded eGFP expression areas were observed with BioAAV2, when compared to the ones obtained with unmodified rAAVrh10 (10 mm^2 *vs* 2 mm^2 for the striatum, 2mm^2 *vs* 0.2 mm^2 for the cortex and 4 mm^2 *vs* no transduction for the thalamus).

Our findings demonstrate that the bioconjugation of lysine or tyrosine onto rAAV vectors provides a promising avenue, complementing genetic engineering methods, to improve protein expression within specific tissues. This strategy holds significant potential for advancing gene therapy interventions targeting for example glaucoma, optic neuropathies, as well as neurodegenerative disorders.

P0147

Selection of tumor targeting adeno-associated viral vectors in an *ex vivo* perfusion model on human cancer patient tissue

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The treatment of cancer has been a major field of research for decades but it remains one of the biggest clinical challenges. In the European Union, cancer is the second most common cause of death, showing the urgent need for improved cancer therapies. With an increasing number of gene therapy approaches targeting cancer getting approved for clinical use, gene therapy offers promising possibilities for supplementing conventional cancer therapies. As viral vector-based gene transfer shows a higher delivery efficacy than gene transfer with non-viral vectors we aim to develop adeno-associated viral (AAV) vectors that specifically target solid human tumors. In order to alter the natural tropism of the AAVs, we incorporated random peptide libraries into the sequence of the capsids of AAV9 and AAV2 at position A589 and R588, respectively. The generated AAV display peptide libraries each yielded above $1x10^8$ individual clones (counted as bacterial colonies after molecular cloning) which subsequently undergo a three-step selection process. To be able to identify AAV variants of clinical relevance, an *ex vivo* screening approach is performed in perfused human organs. Specifically, we use resected human colon and kidney tissue from colon and kidney cancer patients directly after surgery for the *ex vivo* AAV selection. The entire selection process involves three selection rounds with pre-defined numbers of perfused organs per round and with organs from different sexes. Following perfusion, the DNA of the enriched AAV variants is isolated from healthy and tumor tissue and a secondary AAV library is generated after each selection round from the viral DNA found in the tumor tissue. Furthermore, NGS analysis is used to determine the most strongly enriched and most tumor-specific AAV variants. Preliminary data indicates that the diversity of the initial AAV libraries can be reduced by a factor of $1x10^3$-10^4 in one selection round. In addition, we observe a tendency of selected particles found in colon tissue to bind specifically to endothelial cells. The resected organs allow due to their physical condition only one perfusion cycle for colon tissue and up to three perfusion cycles for kidney tissue. For the perfusion procedure, we found that the number

of re-infusion cycles doesn't seem to have a significant effect on AAV enrichment at least when comparing the viral genome analyses of colon tissue with kidney tissue samples. After three selection rounds, the most promising variants obtained as well as natural AAV serotypes and previously identified reference capsid mutants will be produced as a barcoded library and evaluated in the *ex vivo* perfusion system. The best variants will finally be tested for their capacity to mediate tumor-specific gene expression with the aim of establishing cytotoxic or immune modulatory anti-tumor gene therapies.

P0148

AAVrh10 and AAVrh74 Schwann cell targeted gene replacement therapy for CMT1X demyelinating neuropathy

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Adeno-Associated Vectors (AAVs) are promising tools for gene therapy targeting the peripheral nervous system (PNS) to treat demyelinating neuropathies. Previous studies have shown therapeutic benefit of AAV9-mediated gene replacement in mouse models of the second most common form of Charcot Marie-Tooth (CMT) disease, X-linked type 1 (CMT1X). CMT1X results from a loss of function mutations in the *GJB1* gene which encodes the gap junction protein connexin 32 (Cx32). Cx32 is highly expressed in Schwann cells throughout the PNS, forming gap junctions in non-compact myelin areas. In this study we tested two alternative, clinically relevant AAV serotypes, AAVrh10 and AAVrh74, to transduce myelinating Schwann cells in order to develop a gene replacement therapy for CMT1X.

AAVrh10-h*MPZ.EGFP* and AAVrh74-*hMPZ.EGFP* (mock vectors) were delivered by lumbar intrathecal injection in 2-month-old wild type mice (WT) mice at three different doses: 1) $2x10^{11}$ vector genomes (vg), 2) $4x10^{11}$ vg and 3) $8x10^{11}$ vg. The corresponding therapeutic vectors, AAVrh10-h*MPZ.GJB1* and AAVrh74-h*MPZ.GJB1*, were similarly delivered into 2-month-old *Gjb1*-null mice, a model of CMT1X, at the same doses. Mice were sacrificed 6 weeks post-injection for biodistribution and EGFP or Cx32 expression analysis in WT and *Gjb1*-null mice, respectively. Subsequently, we performed a dose-escalation treatment trial in 2-month-old *Gjb1*-null mice with evaluation of functional and morphological outcomes compared to mock-vector treated littermates.

Biodistribution and expression analysis of the mock vectors showed widespread targeting of the PNS and CNS, with dose-dependent increase in vector genome copy numbers (VGCNs) ranging from 6.8-18.9vg (vector copies/host cell) for AAVrh10 and 4.9-16.23vg copies/cell for AAVrh74 in lumbar spinal roots. Similar results were obtained in the other PNS tissues showing a gradient biodistribution from the injection site to the distal part of the nerve. In sciatic nerves, VGCNs ranged from 0.44-7 vg for AAVrh10 and 0.59-9 vg for AAVrh74, including proximal and distal parts. EGFP expression analysis showed similar expression rates in Schwann cells in all PNS tissues examined ranging from 36-50%, showing no significant differences between the three doses. Biodistribution and expression analysis of the therapeutic vectors showed a similar PNS biodistribution and dose-dependent Schwann cell transduction with higher expression levels in

sciatic nerves of *Gjb1*-null mice with both serotypes. Cx32 expression rates in Schwann cells reached 54-67% in lumbar roots and 66-72% in sciatic nerves with no differences between the two serotypes at all doses examined. A subsequent dose-escalation treatment trial in 2-month-old *Gjb1*-null mice including groups of littermate mice injected with the therapeutic or the mock vector at the different doses. Behavioral testing at 2- and 4-months post-injection showed improved grip strength, and electrophysiological evaluation demonstrated improved sciatic nerve motor conduction velocity in treated as opposed to mock-treated CMT1X mice.

This study demonstrates that a single lumbar intrathecal injection of clinically relevant doses of AAVrh10-h*MPZ.GJB1* and AAVrh74-h*MPZ.GJB1* viral vectors leads to adequate PNS biodistribution and high rates of Schwann cell-specific therapeutic gene expression, as well as significant therapeutic benefit in a model of CMT1X demyelinated neuropathy.

AK and KAK contributed equally

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P0149

Anellovectors: encapsidating anellovirus-based vectors beyond the wild-type genome capacity and demonstrating 12 month durable expression of an eGFP transgene post subretinal administration

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Anelloviridae is a family of non-enveloped viruses with negative-sense, circular, single-stranded deoxyribonucleic acid (ssDNA) genomes that infect vertebrates and are a ubiquitous component of the human virome. Human anelloviruses evade induction of humoral immune responses and appear to be non-pathogenic. These properties, in conjunction with their enormous genomic diversity and wide tissue distribution, make anelloviruses compelling candidates as vectors for next-generation genetic medicines. Here we report the first gene delivery vector system based on a human commensal virus: the Anellovector. The Anellovector is based on a virus of the *Betatorquevirus* genus. Production is enabled by the development of the Self-Amplifying Trans-complementation of a Universal Recombinant aNellovector (SATURN) system, which relies on a self-replicating plasmid to provide viral proteins in trans that drive replication and capsid-dependent packaging of vector genomes. The SATURN system also utilizes a Cre-lox-based recombination mechanism to generate single unit-sized circular genomes inside the MOLT-4 production cell line. Anelloviruses are the only truly negative-sense ssDNA viruses that infect eukaryotes. Notably, anelloviruses lack the Rep proteins produced by other ssDNA viruses, pointing to a unique replication and packaging mechanism that may allow for the encapsidation of larger genomes. The Anellovector presented here is based on the Beta genera of human anelloviruses which have genome sizes of ~2.8-3.0 kb. Using vector genomes of increasing size for encapsidation, we have demonstrated that Anellovectors are capable of packaging vector genomes ranging from 2.0kb - 5.0kb, representing up to 65% beyond the wild-type genome capacity. The Anellovector demonstrated function *in vitro* using both HEK293TT cells and retinal

pigment epithelial (RPE) cells, the cell type from which this betatorquevirus was originally discovered. We next investigated the potential of the Anellovector to serve as platform capable of durable gene expression. Using an eGFP transgene to track expression, the Anellovector demonstrated durable *in vivo* expression in the mouse eye for 12 months following subretinal administration. Anellovectors have great potential to deliver safe, durable, redosable, and potent therapeutics, helping to expand the reach of programmable medicines.

P0150

AAV-Boost: enhancing the transduction of specific target cells with bispecific nanobody adaptor proteins

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AAV vectors are useful gene delivery tools. However, their low cell specificity and off-target effects remain crucial limitations for therapeutic applications. Nanobodies are the variable immunoglobuiln domains of camelid heavy chain antibodies. Due to their small size, high solubility, and easy reformatting options, they are valuable tools in research and therapy. We have previously shown that the insertion of a membrane protein-specific nanobody into the capsid of an AAV vector can drastically increase the transduction of cells expressing the respective target protein (1). Here we describe an alternative strategy to boost AAV transduction of specific cells using bispecific nanobody adaptors. In order to generate AAV-specific nanobodies, we immunized llama-IgH transgenic mice (LamaMice) and selected a variety of AAV-specific nanobodies (2). We genetically fused each AAV-specific nanobody to a membrane protein-specific nanobody via a flexible glycine-serine linker. Some of these bispecific adaptor proteins dramatically boosted AAV transduction of target cells expressing the respective membrane protein. Due to their modular composition, the adaptors can easily be adjusted to different target proteins and AAV serotypes. We provide proof of principle for the utility of these AAV boosters for a variety of cell surface proteins, including transmembrane and GPI-anchored ecto-enzymes. It remains to be determind whether this technology can also be used to lower AAV-dosing, treatment costs and off-target effects for *in-vivo* therapies.

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2) Eden, T., Schaffrath, A.Z., Wesolowski, J. et al. Generation of nanobodies from transgenic ‘LamaMice’ lacking an endogenous immunoglobulin repertoire. Nat Comun 15, 4728 (2024).

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P0151

Evolving Adeno-Associated Virus 9 (AAV9) for brain targeting: from *in vivo* screening to receptor-based pull-down

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AAV9 is a delivery platform highly exploited to develop gene-based treatments for neurological disorders given its low pathogenicity and brain tissue tropism. However, the efficacy of this vector is dampened by its relatively low efficiency to cross the adult Blood Brain Barrier (BBB) and inherent targeting to the liver upon intravenous delivery. More recently new AAV9 variants has been discovered that can cross the BBB in mice but in Non-Human Primates (NHP) are less effective. The reason of specie-specificity relies on small glycosylphosphatidylinositol (GPI) anchored proteins that are not conserved in primates. Using a AAV9 display peptide library we executed two different type of screenings: an *in vivo* approach in mice defective for a murine GPI proteins (BALB/c), paralleled by a novel pull-down assay, based on immobilization of specific membrane proteins, in order to tailor capsid variants on a desired receptors. The peptide library was inserted in the AAV9 capsid with additional W503A mutation, which is known to erase the AAV9 natural biding on galactose thus facilitating new interactions. Our first *in vivo* selection identified two enriched capsid variants that were named AAV-Se1-A503 and -Se2-A503. *In vivo* characterization of these variants, in two mice strains, confirmed their brain tropism, that was enhanced restoring galactose binding, trough the reversion of the W503A mutation. The two new capsids were named AAV-Se1 and -Se2 respectively and further characterized *in vivo*, not only in mice but also in NHP, where they displayed an enhanced ability to transduce neurons and glia, compared to their natural variant: AAV9. Nonetheless, further analysis of these variants revealed that AAV-Se2 but not AAVSe1 cells transduction partially depends on a GPI-protein not conserved in primates. This evidence prompted us to revolve our capsid selection approach, focusing on receptor binding rather in vivo brain targeting. To this purpose we chose to target a GPI protein very well conserved between rodents and primates. Variants selected in this screening are currently under *in vitro* and *in vivo* characterization. The outcome of AAV capsid selection strongly depends on the approach employed to screen it. Although *in vivo* screens ensure tissue targeting and biodistribution they can be misleading in terms of cell-specificity and specie-specificity, which instead can be pursed using receptor-based screens.

P0152

Orthogonal Characterization of rAAV Reveals Vector Attributes and Capsid Interactions that Drive ITR Repair and Self-Complementary Genome Formation

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Recombinant adeno-associated viruses (rAAVs) are the flagship vehicles for delivering DNA payloads for human gene therapy. However, in the past decade, only a few outstanding therapies have reached the market. One reason for the slow development of rAAV-based gene therapies has been our limited knowledge of basic AAV biology. Therefore, the goal of this work is to perform orthogonal evaluation of design elements within the packaged rAAV genomes and assess their impact on potency. Specifically, we investigated the influence of inverted terminal repeat (ITR) design, promoter use, and serotype on vector heterogeneity and transduction.

We first analyzed the integrity of AAV-*Egfp* vectors containing either an alpha-1 antitrypsin (*AAT*) promoter (2 kb in vector genome size) or a chicken beta-actin (*CBA*) promoter (3 kb in size), packaged with six different serotypes (AAV2, AAV3B, AAVLK03, AAV5, AAV8, and AAV9) by native agarose and automated electrophoresis. In addition to the bands at the expected genome size, additional larger and smaller bands were also identified, most of which were eliminated when the vector genomes were digested with SmaI, suggesting these DNA species were unresolved and bound at the ITRs. Banding patterns were more heterogeneous with vectors harboring the *CBA* promoter, especially for those packaged with AAV8. We next produced constructs flanked by different ITR designs: two wildtype (wt)ITRs, a commercially available 11-nt deletion in the C arm of the 5'-ITR (dITR), or a 22-nt deletion in the B arm of the 3'-ITR (tITR). Alkaline gel analysis revealed monomer and dimer genomes for AAV3B, AAV5, AAV8, and AAV9 packaged vectors that carried the wtITR and dITR. However, only dimers were observed for vectors packaged with dITR-AAVLK03 and tITR-AAV8, suggesting a role for the capsid in genome replication. In addition, genome heterogeneity was confirmed for the dITR.AAV8.*CBA.Egfp* vector, but not for the dITR.AAVLK03 or dITR.AAV5.*CBA.Egfp* vectors; although, the three vectors showed an intriguing faint band larger than 5 kb.

To validate these findings, long-read single molecule, real-time (SMRT) sequencing was performed on the vector genomes. Our analyses confirmed the presence of genome dimers with self-complementary configurations, including vectors over 3 kb in size. To determine whether the detection of oversized genomes was due to artifacts from target ligation/recombination during SMRT library preparation steps, 2-kb and 3-kb vector genomes were extracted and mixed before library preparation. Clear individual peaks and their respective self-complementary forms were detected, with no sign of recombination between the two genomes. Furthermore, mutated ITRs have been shown to be repaired during rAAV genome replication and packaging, using the more functional of the two ITRs as a template. Interestingly, our bioinformatic analyses revealed that the genome length has an impact on ITR repair. Finally, *in vitro* transduction studies showed superior transgene expression by vectors with higher proportions of self-complementary species. In conclusion, self-complementary genome encapsidation is favored under specific ITR designs that can be influenced by other elements such as the promoter or the AAV capsid.

P0153

Advancements in nanopore sequencing allow in-depth characterization of rAAV vector batches comparable to SMRT™ sequencing

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Genetic payloads of recombinant adenovirus-associated viruses (rAAVs) often exhibit heterogeneity, including undesired DNA impurities and truncated vector genomes. Long-read next-generation sequencing enables a thorough analysis of encapsidated DNA that is crucial for guiding platform optimization and ensuring the safety and efficacy of gene therapies. In this study, we used nanopore sequencing (Oxford Nanopore Technologies) to conduct a comprehensive profiling of a rAAV9 batch produced using a proprietary split plasmid system in a 50-liter bioreactor.

We compared three different methods for converting single-stranded to double-stranded DNA and assessed their impact on sequencing data. Our analysis revealed distinct library size profiles among the methods, but comparable encapsidated DNA impurity distribution. Additionally, we compared recent advancements in nanopore sequencing, such as the V14 chemistry and dorado basecalling software, with the widely used V9 chemistry and guppy basecalling software. We observed a substantial increase in raw read quality and alignment accuracy with the latest methods.

Our data demonstrated overall high vector quality (>95% reads mapping to the vector), with notably very low levels of host cell DNA (HCD) impurities, which were primarily shorter than 1000 nucleotides and of random origin. These data are crucial for mitigating associated risks for example in regulatory submissions.

The same rAAV batch was sequenced using SMRT™ sequencing (PacBio), to compare our high quality nanopore data to the current gold standard of AAV sequencing. The percentage of reads mapping to HCD and the helper plasmid was nearly identical compared to nanopore sequencing. In contrast, SMRT™ sequencing resulted in more reads mapping to the plasmid backbone than nanopore sequencing potentially from distinct assignment of chimeric reads, i.e. reads that map to both the vector and the plasmid backbone.

A detailed analysis of the read mapping borders can be used to identify vector truncation hotspots. When we performed a truncation analysis, similar results compared to data acquired by a commercial SMRT™ sequencing service were observed. This confirmed that nanopore sequencing is suitable for this analysis type.

In summary, our results show that nanopore sequencing which is our method of choice enables in-depth characterization of DNA packaged into AAV capsids with data being very comparable to those obtained with SMRT™ sequencing. In addition, nanopore sequencing requires low input amounts and yields more reads with high raw read quality.

P0154

Hacking Biology: Optimizing AAV-Based Gene Delivery for Enhanced Region-Specific Brain Targeting

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Gene therapy has made significant strides using Adeno-Associated Viral (AAV) vectors, prized for their non-toxic, low immunogenicity, and non-integrating traits. These vectors hold promise for treating diverse genetic disorders. However, natural AAV serotypes pose challenges due to limited specificity, prompting researchers to explore engineered viral capsids. This exploration involves studying natural serotype diversity, their tissue tropisms, and refining specificity through innovative design methods like Rational Design, Directed Evolution, and Computer-Aided Design. This study aimed to optimize AAV-mediated gene delivery, emphasizing targeted delivery to specific brain regions via intravenous (IV) injection.

The approach adopted a rational strategy, leveraging published Next-Generation Sequencing (NGS) data from Gradinaru's lab at Caltech to pinpoint optimal capsids for transduction across various brain regions. Python scripts and clustering tools such as MATLAB and Cytoscape analyzed the NGS data to identify top capsid sequences tailored for the Cerebellum, Brainstem, Spinal Cord, Forebrain, Thalamus, and Midbrain. The study linked specific amino acids and their positions in the capsid protein to AAV biodistribution in mouse models, culminating in the identification of the top 25 variants for each brain region. As proof-of-concept, the study selected and produced the top four capsid sequences for targeted brain regions. These vectors encoded luciferase and GFP under the control of the EFS promoter. Adult wild-type C57BL/6 mice and neonates at postnatal were intravenously injected with 1E11 viral particles of each capsid variant via retro-orbital administration. Following injection, animals underwent luminescence analysis using an In Vivo Imaging System (IVIS) at 15- and 30-days post-injection. Subsequently, mice were sacrificed, and viral genome copies in different brain regions and the liver were quantified using quantitative real-time PCR, targeting Inverted Terminal Repeat (ITR) sequences.

IVIS luminescence data indicated that the four novel AAV capsids exhibited enhanced brain transduction in adult mice with reduced liver transduction compared to the natural serotype AAV9. These findings were corroborated by qPCR analysis of viral genomes/DNA content across multiple brain regions. In an initial experiment, focusing on cerebellar, spinal cord, and brainstem transduction enhancement, adult mice injected with new C2 capsid and CAP.B10 capsids demonstrated the highest brain luminescence levels. The study confirmed that new AAV capsids, like CAP.B10, displayed superior brain-targeting efficacy relative to AAV9, with qPCR revealing consistent transduction levels across diverse brain regions.

Distinct luminescence patterns were observed in neonates injected with AAV9, indicating age-dependent variations in transduction efficiency. *Ex vivo* assessments highlighted AAV9's robust brain transduction alongside heightened liver transduction. qPCR data confirmed significant reductions in liver viral genomes for CAP.B10 and new AAV capsids compared to AAV9,

underscoring their potential as superior candidates for liver de-targeting while effectively transducing the brain.

This research underscores the potential of rational AAV design tailored for systemic administration to specific brain regions, promising transformative impacts on therapeutic strategies for neurodegenerative diseases.

P0155

Development of novel high-resolution multiplex digital PCR (HR-mdPCR) approach for detailed evaluation and quantification of vector genome or DNA impurities sequence integrity

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In the production of viral vectors for gene therapy, the emphasis is on pure, safe, and effective products. The absence of impurities and the presence of a complete vector genome play a crucial role. If the viral genome is not complete, no therapeutic effect or a much lower therapeutic effect can be expected; moreover, such fragments, or presence of DNA impurities, may generate neoantigens in cells leading to unexpected immune responses. The integrity of the viral vector genome or other sequences can be assessed by several methods, each of which has its advantages and disadvantages, however long-read sequencing and multiplex digital PCR are the most common sequence specific technologies. Nevertheless, long-read sequencing is not truly quantitative, and multiplex digital PCR does not provide information on presence/absence of sequences between the targeted regions. To enable sequence integrity analysis, which would overcome the time and price challenge of long-read sequencing, but at the same time provide an absolute quantification of longer targets, we have developed a novel approach called high-resolution multiplex digital PCR (HR-mdPCR), which provides a detailed insight in the sequence integrity of lengths up to 4.6 kb. We have initially developed the 7-plex assay to cover the whole length of the nptII (kanamycin resistance) gene, thus providing high-resolution quantitative insight in the integrity of its sequence. This idea will be further extended to longer sequences, such as full length AAV viral vector genomes. By using HR-mdPCR we can obtain results on sequence integrity that are in a level with long read sequencing, but providing a quantitative value in a time that is much shorter than by sequencing. This technology has the potential to replace sequencing in several stages of vector development and manufacturing processes where analytical characterization is required.

P0156

An updated nanopore sequencing protocol for characterization of contaminants in rAAV vector preparations

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Manufacturing of rAAV particles for gene therapy leads to a significant fraction of particles not containing the full length ITR-to-ITR transgene cassette. These contaminants include vectors packaging truncated variants, host cell or plasmid DNA, or no DNA at all, which reduces the potency of vectors and might lead to toxicity or immunological responses. Current AAV analytical methods have mainly focused on quantification of full, partially full or empty particles. However, the ability to specifically characterize sequence information of the packaged DNA is essential to gain a comprehensive understanding of AAV gene therapy products' safety and efficacy. Long-read sequencing technologies such as nanopore DNA sequencing offers read lengths that are very well suited for end-to-end sequencing of rAAV DNA content. However, the ITRs' secondary structure, the single strand nature of the DNA and the need for sequencing adaptor ligation pose challenges for this otherwise easy-to-use and cost-effective method. Additionally, careful bioinformatic interrogation of the resultant sequencing reads is crucial for correct data interpretation. Here, we have optimized several key steps in the library preparation procedure for nanopore sequencing, including DNase treatment, viral DNA extraction, and viral genome annealing protocol. Moreover, we present a simple, user-friendly, and easily interpretable bioinformatic workflow for characterizing the sequence identity of the rAAV-packaged DNA. We believe that the ease of use, cost efficiency and depth of information afforded by nanopore sequencing will enhance rAAV manufacturing practices, ultimately improving safety and efficacy of AAV gene therapy products.

P0157

Streamlined pre-formulation screening with minimal sample requirements and a turnaround time of 2 days enables development of AAV formulations with high efficiency

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Material constraints are a limiting factor in early stage AAV gene therapy development programs. This is especially true for initial, broad formulation studies, that consume substantial amounts of material and time. We have established a low-volume, high-throughput pre-formulation screening approach using a **simple proprietary buffer matrix** that spans a broad pH range (5 – 8) and ionic strength levels (using 100 – 310 mM NaCl). We show that rebuffering of AAV using a **commercially available 96-well spin-plate** loaded with size-exclusion resin provides a rapid and reliable means of rebuffering. With only **200 µL per condition**, the material from this

rebuffering campaign was used to generate thermal (nanoDSF) and colloidal (DLS) stability data along with assessing subvisible/visible particle content (BMI), DNA ejection (using SYBR GOLD) and titer recovery (Stunner). Since **all methods are compatible with the 96-well format**, results from this screening approach are available within hours of the rebuffering procedure with minimal manual handling. For the exemplary serotype used in this study, a buffer at pH 8 (20 mM TRIS, >190 mM NaCl) initially provided satisfactory results, which is in line with published data. However, **DNA ejection progressed steadily** at the tested temperature (37°C) over 2 days **compared to all other tested buffers**, which exhibited a much **less pronounced payload release.** Thus, development of a liquid formulation for the serotype in question should focus on buffers at lower pH levels to mitigate loss of encapsulated DNA during storage.

Overall, the wealth of data generated from this method with **just 8 mL of sample AAV** can provide meaningful insights towards buffer and ionic strength preferences of a given serotypes and further inform rational formulation development. The approach is capsid agnostic and can be applied to any AAV serotype or other modalities based on protein nanoparticles.

P0158

In vivo selection of an AAV2-*eGFP*-barcode library identifies two novel capsid variants with high specificity for cardiomyocytes

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Adeno-associated viral (AAV) vectors are promising *in vivo* gene delivery tools due to their low immunogenicity and stable gene expression in non-dividing cells. In particular, AAV vectors hold great potential for the treatment of cardiovascular diseases, which are among the leading causes of death worldwide. While several AAV gene therapy products have already been approved for clinical use, a safe and efficient gene therapy for the heart is still missing. AAV serotype 9 (AAV9) is commonly used for *in vivo* transduction of cardiomyocytes in rodents. However, the high off-target expression in the liver restricts the application of AAV9 vectors for cardiac gene therapy in clinical settings due to the risk of hepatotoxicity. In previous studies, we reported on two novel highly promising heart-specific capsid variants AAV2-THGTPAD and AAV2-NLPGSGD derived from an *in vivo* AAV2 peptide display library screening in a mouse model of pressure-overload cardiac hypertrophy. For further in-depth analysis of our previous *in vivo* screen, we equipped the top 53 cardiomyocyte-specific candidates with a barcoded *eGFP* expression cassette for a direct *in vivo* comparison. We thereby identified two capsid variants that showed an even higher transduction efficiency as compared to AAV2-THGTPAD and AAV2-NLPGSGD, while leading to only a minor transduction of liver tissue. Analysing the two novel variants as individual AAV vector variants expressing *eGFP* confirmed cardiac tropism as indicated by an improved cardiomyocyte-to-liver transduction ratio as compared to the gold standard vector AAV9 (2-fold and 4.9-fold, respectively). *In vitro* assays demonstrated that the retargeting of the vectors is accompanied by a reduced binding affinity to the AAV2 primary receptor heparan sulfate proteoglycan. We could not detect any cross-neutralizing antibodies between wild type AAV9 and our capsid variants, which enables a combined use of AAV9 and our novel vectors for repeated injections. Interestingly, one of the capsid variants showed a disease-specific tropism and was inefficient in transducing cardiomyocytes of healthy mice. We are following up this interesting feature with experiments in mice after transverse aortic constriction (TAC) as well as in a *Mybpc3*-mutant mouse line with hypertrophic cardiomyopathy. Discovering novel AAV

vectors that target cardiomyocytes, particularly those affected by disease, will improve the safety and feasibility of cardiac gene therapies, thereby enhancing the treatment options for many patients with inherited and non-inherited cardiac diseases.

P0159

Evaluating the permissibility of human iPSC-derived cell types to AAV transduction as an in vitro model for gene therapy

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INTRODUCTION: The differentiation of induced pluripotent stem cells (iPSC) into specialized cell types of the human body represents a major advancement for the development of biologically relevant disease models. Generation of patient-specific cell models for in vitro testing has cultivated the concept of “disease-in-a-dish” for iPSC-based disease modeling. These model systems are increasingly being used to evaluate various gene targeting approaches, including adeno-associated viral vectors (AAV), to directly correct the genetic defect or mutation. The use of AAV for gene therapy has recently become a clinical reality for the treatment of spinal muscular atrophy (SMA) and a rare form of blindness. To identify relevant iPSC-based in vitro models to support the analytical characterization of AAV gene therapies, the permissibility of different iPSC-derived cell lines to specific AAV serotypes needs to be defined. This requires the establishment of an efficient protocol for the AAV transduction of a particular iPSC-derived cell type for which several aspects need to be optimized, including viral vector serotype, multiplicity of infection (MOI), promoter, transduction media, and timing of transduction.

METHODS: Human iPSC-derived cell types are commercially available: iCell® Cardiomyocytes (iCell CM) and iCell Retinal Pigment Epithelial Cells (iCell RPE) from FUJIFILM Cellular Dynamics, Inc. (FCDI). Cryopreserved cells were thawed, plated, and maintained in culture according to the manufacturer’s recommendations until the time of transduction. AAV viruses engineered to express green fluorescent protein (GFP) were prepared internally or obtained commercially. Different methods to evaluate transduction efficiency included standard fluorescence microscopy to visualize GFP-positive cells, flow cytometry (CytoFLEX, Beckman Coulter) to quantify green cells, and Celigo image cytometer (Nexcelom) to both obtain images and quantify GFP in situ.

RESULTS: iCell CM and iCell RPE were transduced with AAV1, -2, -4, -5, -6, -8, and -9 in standard maintenance medium and serum-free medium on days 7 and 10 post-thaw for iCell CM and days 14 and 21 post-thaw for iCell RPE. AAV6 showed the highest transduction efficiency in iCell CM, with AAV2 also working well. AAV2 show high transduction efficiency in iCell RPE. However, the capability of other AAV serotypes to transduce iCell RPE remains to be tested. In general, transduction on the earlier timepoint, day 7 for iCell CM and day 14 for iCell RPE, worked better than on the later timepoint, day 10 for iCell CM and day 21 for iCell RPE, with some exceptions. Moreover, the CAG promoter drove higher transgene expression than CMV. In addition to vector tropism, comparison of iPSC-CM and iPSC-RPE show that cell type significantly affects transgene expression.

UPDATE: We have also collaborated with Promega to evaluate parallel (non-fluorescent) approaches to screening AAV serotypes for transduction efficiency and tropism across

iCell products. Importantly, it was demonstrated that AAV6 also yielded the highest performance in iCell CM.

P0160

Translocation Event Detector in rAAV (TED-rAAV), A tool for detecting rAAV structural dysregulations and large genomic insertion/deletions with high sensitivity and accuracy

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The recent positive outcomes observed in clinical trials utilizing recombinant adeno-associated viral vectors (rAAVs) have reignited scientific enthusiasm for gene therapy. While substantial progress has been made in enhancing the manufacturing procedures of vectors, the persistence of unwanted DNA impurities in rAAV formulations continues to pose a significant safety apprehension. Particularly, the inherent translocation events during rAAV production or the loss of crucial functional regions within the rAAV genome pose challenges for current contamination detection methods. To address this, we introduce TED-rAAV, the rAAV Translocation Event Detector, which leverages a Hidden Markov Model (HMM) approach in conjunction with translocation breakpoint information identified in sequencing fragments. This detector demonstrates the capability to accurately identify long deletion events and recombination events exceeding 200 base pairs within the AAV genome with a detection sensitivity of down to 1%. Moreover, it can detect recombination events between AAV and other sources of contaminating DNA, such as AAV-human genome hybrids. These progressions are set to establish the foundation for a fresh phase of quality checks, guiding the refinement of procedures towards creating rAAV products with stronger effectiveness and safer characteristics.

TED-rAAV was designed as a hybrid algorithm employing a priori informed HMM to enhance the detection sensitivity and precision in translocation and long-range detection in recombinant AAV based on NGS data. HMM naturally excels in detecting sequential disorders, its algorithmic specificity prevents it from precisely determining genome breakpoint locations at the base pair (bp) level. TED-rAAV modifies HMM by adjusting the transition probabilities near breakpoint positions based on the breakpoint locations obtained from sequencing fragments. This modification can greatly enhance the break-point detection accuracy of the HMM algorithm by 4 folds, from a median deviation of 104 bp (19bp to 214bp) to 5 bp (2bp to 6bp) in 24 samples tested.

TED-rAAV was trained by learning the sequencing features in pure AAV and AAV with translocations and long deletions in the genome. We employed a grid search technique to optimize the parameter settings in the HMM algorithm. In TED-rAAV, when a breakpoint was detected, the transition probabilities in an HMM near the breakpoint will be elevated to ensure that the model accurately captures the starting points of a state transition. To verify the performance of TED-rAAV, we mixed rAAV containing known genomic deletions (1000-1500bp) with pure AAV at varying concentration gradient ratios ranging from 20% to 1%, and sequenced it by more than 100k in depth. We found that that TED-rAAV can precisely determine the deletion status even at concentrations as minimal as 1% and can estimate the percentage of AAVs with segment deletions by assessing the ratio of translocation fragments in a semi-quantitative manner.. In contrast, the orthogonal HMM algorithm is only capable of detection at a

concentration level of 5%. Furthermore, when incorporating translocation site information, TED-rAAV could precisely locate the deletion sites at the base pair level (deviation between 2-6 bp), with a positional discrepancy of no more than 10bp from the true positions, surpassing the accuracy achievable by current HMM algorithm.

P0161

Novel nanofiber-based microcarriers designed for improved upstream productivity of viral vector biomanufacturing

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Viral vector manufacturing needs to ensure scalability while upholding high product yield, quality, and process robustness. Adherent cell cultures systems known for their ability to produce high yields of viral vectors are associated with scalability challenges. Nanofiber-based microcarriers, a novel microcarrier format, providing three-dimensional (3D) cell culturing of adherent cells readily scalable in stirred tank (suspension) bioreactor systems, offers promising avenues for scale up of adherent cell cultures. Data shows that HEK293T cells adhering to the nanofiber-based microcarriers form homogenous 3D cultures with high cell viability. In addition, the microcarriers large surface area and lightweight nature allow for achieving high cell densities and reduced shear forces in bioreactors. Importantly, transient transfection of adherent HEK293T cells growing on the nanofiber microcarriers shows high (>90%) transfection efficiency and volumetric productivity (10^{14} vg/L) of human adeno-associated virus serotype 2 (AAV2) vectors. These findings demonstrate the potential of the nanofiber-based microcarriers as a scalable platform for adherent cell cultures in gene therapy applications, advancing bioprocessing strategies for enhanced yield, reduced time and costs leading to improved upstream bioprocessing productivity.

P0162

AAV5-vector DNA clearance in semen after etranacogene dezaparvovec gene therapy in haemophilia B: Findings from the HOPE-B trial

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Although adeno-associated virus (AAV) vectors are replication incompetent and thus pose minimal risk for transmission to third-parties or upon release into environment, a comprehensive assessment of vector shedding (release and presence of vector DNA in bodily fluids) is required, as part of the gene therapy clinical development program's safety outcomes. Notably, the data relative to vector DNA presence in semen may be used by regulatory agencies to develop drug use recommendations, such as suitability or duration of barrier contraception. Vector shedding in

semen was evaluated in an ongoing pivotal Phase 3 HOPE-B trial (NCT03569891) following etranacogene dezaparvovec (CSL222, formerly AMT-061, HEMGENIX®) administration in patients with severe and moderately severe haemophilia B.

In the HOPE-B trial, 54 adult males with severe or moderately severe haemophilia B received a single intravenous infusion of 2×10^{13} gc/kg of etranacogene dezaparvovec, an AAV5 vector containing the factor IX [FIX] Padua (R338L) transgene under the control of the liver-specific promoter LP-1. Vector DNA measurement in semen was performed using a validated qPCR assay. Semen samples were collected until vector DNA clearance was obtained, defined conservatively as 3 consecutive negative results below limit of detection (below LOD via a specific qPCR, as per ICH guidelines). Samples were collected maximum at Week 6 (earliest), Week 12, Month 4, Month 6, and then every 6 months up to 5 years post-treatment. Vector DNA presence ("shedding positive") was defined as any sample above LOD (even if lower than the limit of quantification [LLOQ]); vector DNA absence ("shedding negative") was defined as first of 3 consecutive qPCR determinations below LOD, when data were available.

Following etranacogene dezaparvovec administration, vector DNA was detected in all participants (excluding 2 who refused to provide any study semen samples). Peak vector DNA concentrations in semen following etranacogene dezaparvovec administration were observed early and decreased rapidly. Median (95% CI) time to vector shedding negative was 43.7 weeks (34.1–51.9). No semen sample collected contained quantifiable (above LLOQ) vector DNA from Month 5 post-treatment, and no semen sample collected was positive (above LOD) for vector DNA presence from Month 13 post-treatment; 36/54 (67%) were considered shedding negative at year 1, and 45/54 (84%) at year 2 post-treatment (representing all 45 participants who provided semen samples at this study stage).

These data highlight the challenge of missing data for the time interval between the last confirmed positive and quantifiable value (above LLOQ), and the first point returning to negative (below LOD). The detected shedding signal is most likely due to fragmented vector DNA and does not equate to transduction competent vector particles. Interpretation of vector DNA clearance data varies and, depending on detection methods and definitions of vector clearance, may lead to variation in patient advice in terms of barrier contraception duration during the immediate period post-treatment. Based upon the replication incompetent nature of etranacogene dezaparvovec and the maximum potential exposure to the vector in semen following etranacogene dezaparvovec administration, the risk of transmission to untreated individuals is considered extremely low and remains theoretical as supported by preclinical data from the literature.

P0163

Impact of pre-existing immunity on biodistribution and safety of a single intrathecal injection of an AAV9-based gene therapy in cynomolgus monkeys

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Adeno-associated virus (AAV) vectors are widely used for gene therapy applications, as they can deliver therapeutic genes with minimal toxicity and immunogenicity.

The presence of pre-existing anti-AAV immunity in a large proportion of the human population, due to natural exposure to AAV infections, remains one of the major challenges of AAV-based gene therapies. Pre-existing immunity can reduce the efficacy and safety of AAV-based gene therapies, as it can lead to neutralization of the vector, clearance of the transduced cells, and inflammatory responses. Consequently, only patients with no or very low vector-specific pre-existing antibodies are currently eligible for most of the available AAV-based gene therapies and inclusion in clinical trials.

Here, we investigated the effect of different serum levels (low, medium, and high) of pre-existing anti-AAV9 antibodies on the biodistribution and safety of a scAAV9-mCherry vector (1.2 $x10^{13}$ vector genome/animal), delivered to cynomolgus monkeys with a single intrathecal injection. Predose, all animals had no or very low anti-AAV9 antibody in the cerebrospinal fluid (CSF) when compared to serum.

High serum levels of AAV9-specific antibody did not impact vector genome biodistribution to the central nervous system tissues (e.g. the spinal cord and brain), but diminished vector genome biodistribution to the dorsal root ganglia, liver, and other peripheral tissues, except for the spleen where vector genome copies increased.

Treatment-emergent antibody responses against AAV9 capsid were observed in all immunized animals. However, animals with medium and high pre-existing antibodies developed higher serum levels of therapy-induced antibodies already at day 8 post-injection when compared to the low titer group, due to a stronger contribution of the memory humoral response. In the CSF, therapy-induced antibodies were also measured but at lower levels compared to serum. Higher AAV9-specific T cell responses, mainly CD4, were observed in the spleen of immunized animals with a higher level of pre-existing anti-AAV9 antibody 4-week post injection, in line with the contribution of memory T cells and the increased vector biodistribution in the spleen. Clinical pathology and chemistry as well as pathology assessments excluded major safety concerns for all animals over a 4-week period.

Altogether, the biodistribution and safety data from this NHP study suggest that using anti-AAV antibody titers as a criterion for patient eligibility for intrathecal AAV-based gene therapy could lead to the unnecessary exclusion of patients who may benefit from the treatment. The observed stronger anti-vector responses in the presence of high levels of pre-existing vector-specific antibodies suggest a potential need, if used in this patient population, to closely monitor the therapy-induced immune response and potential consequences.

P0164

Co-delivery of AAV based RPE65 and Survivin augments visual response in preclinical models of LCA2

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Leber congenital amaurosis type 2 (LCA2) is a hereditary retinal disorder causing severe vision impairment in children. Gene therapy-based products using Adeno-associated virus (AAV) serotype 2 delivery of the human *RPE65* gene are available, but the long term follow-up studies in

patients, have shown a gradual decline in vision 3-5 years after gene therapy. To address this limitation, we investigated a combination gene therapy approach, to enhance vector characteristics and to improve retinal health by using an anti-apoptotic factor, Survivin. We compared the efficacy of modified RPE65 transgenes (Kozak/ codon optimized [CodOpt]) carried by a capsid modified AAV2 vector (AAV2K665Q) against wild type RPE65 (RPE65WT). In human retinal cells (ARPE19), the CodOptRPE65 vector exhibited a 1.8-fold increase in RPE65 protein expression in comparison to the RPE65WT vectors. We then investigated the therapeutic impact of this strategy, by subretinal gene transfer of AAV2K665Q- CodOptRPE65 vector co-delivered with AAV5-Survivin vectors, in a pre-clinical (rd12) mouse model of LCA2. Animals were monitored for up to 6 months, with electroretinography showing 2.57- fold and 1.76- fold improvement in A- and B-wave amplitudes, respectively, in combination treated eyes (CodOptRPE65+ Survivin). Functional assessment using Morris water maze test indicated that combination vector treated mice reached the platform in approximately half of the time in comparison to the mock-treated animals. Immunohistochemical analysis revealed notable RPE65 expression, with no vector induced immune response as indicated by glial fibrillary acidic protein (GFAP) expression in combination treated eyes. Additionally, the expression of Bax, a pro-apoptotic protein, was significantly reduced in animals receiving the combination therapy of RPE65 and Survivin. This data was independently confirmed by a decrease in cellular apoptosis by TUNEL assay in combination treated animals. These findings suggest that incorporating anti-apoptotic factors may strengthen the phenotypic rescue and help manage retinal degeneration in LCA2 patients, offering a promising approach for clinical implementation.

P0165

A dose-escalation and safety study of AAV9 gene replacement therapy for the treatment of PMLD1/HLD2

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Autosomal recessive mutations in the *GJC2* gene encoding the gap junction (GJ) protein Cx47 cause Pelizaeus-Merzbacher-like disease (PMLD1)/ hypomyelinating leukodystrophy type 2 (HLD2). PMLD1 leukodystrophy is an infancy-onset progressive disease, causing severe disability that accumulates over time, having a considerable impact on patient's life and on their families. Cx47 is highly and specifically expressed in all oligodendrocytes throughout the CNS of rodents and humans, forming the majority of oligodendrocyte to astrocyte as well as most inter-oligodendrocytic gap junction channels. In order to treat PMLD1 we developed a cell-targeted GJC2 gene replacement in oligodendrocytes using intravenously injected AAV9 vector carrying the therapeutic gene (human *GJC2*/Cx47) under the control of myelin basic protein promoter (Mbp).

Groups of Cx32/Cx47 double knockout mice, a model of PMLD1, were injected with 3 different doses of AAV9-Mbp.GJC2/Cx47 in order to evaluate biodistribution, gene expression, safety, and therapeutic benefit in the disease model. A mock vector treated group was used as a control. Mice were injected at P10 and evaluated at P30. We examined the extend of Cx47 expression in oligodendrocytes throughout the CNS by immunostaining of brain and spinal cord in longitudinal sections. Furthermore, cell specificity using double staining for cell markers to label oligodendrocytes, neurons, astrocytes and microglia and expression rates (% EGFP positive oligodendrocytes in each CNS area) were evaluated. We found that expression was restricted to

oligodendrocytes, and was not detected in neurons, astrocytes or microglia. High dose provided overall higher expression rates in oligodendrocytes compared to other two doses. Morphometric analysis of myelination was performed in semithin sections of brain and spinal cord by calculating the myelin fraction to estimate the density of myelinated fibers and myelin sheaths. In addition the degree of astrogliosis was determined by quantifying GFAP immunoreactivity and microglia activation was measured by quantifying Iba1 immunoreactivity. Analysis of myelination in corpus callosum, internal capsule, anterior and posterior spinal cord white matter confirmed improved myelin density and reduced vacuolation in all treated mice especially at the high dose injected mice. Furthermore, analysis of inflammatory glia responses showed improvement in astrogliosis and microglia activation in the CNS of treated mice at middle and high dose. Behavioural studies were also performed for comparing the therapeutic groups with mock-treated mice, using the foot slip test, rotarod analysis and foot print analysis. We found significant improvements in motor performance at middle and high treated compared to mock-treated dKO mice. Finally, survival was significantly prolonged in treated compared to untreated dKO animals.

This project provides a proof of principle for a gene replacement approach to treat PMLD1, as well as potentially other leukodystrophy forms resulting from mutations in structural genes expressed by oligodendrocytes.

P0166

An engineered adeno-associated viral vector enabling efficient and transient whole-brain mRNA delivery

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Adeno-associated virus (AAV) vectors are commonly used for DNA delivery in gene therapy applications, and they have several advantages over other viral vectors, including lower toxicity and the availability of over 150 naturally occurring genotypes and serotypes. Therefore, naturally occurring or newly engineered AAV capsids are promising for targeting non-liver tissues, such as the brain, skeletal muscle, and heart. However, using AAVs for *in vivo* gene editing raises safety concerns such as off-target effects, DNA integration, and immune responses to prolonged Cas expression. Messenger RNAs (mRNAs) have emerged as a new category of therapeutic agents to combat a wide range of incurable diseases. However, systemic injection of mRNA-delivering lipid nanoparticles (LNPs) or virus-like particles (VLPs) has limited therapeutic applications due to liver accumulation, a significant barrier in the development of therapeutically efficacious RNA drugs and limitation to deliver drugs to challenging tissue targets such as the brain due to the presence of the blood-brain barrier (BBB). Here, we developed a system enabling the AAV shell to package mRNAs by introducing RNA-packaging components into the AAV production system (triple-plasmid system). We replaced the DNA packaging signal of AAV (inverted terminal repeat, ITR) with RNA packaging signals, which enabled the binding of Rep78/68 proteins to RPS-harboring mRNAs to convert the AAV into an RNA-packaging virus. Our multi-step engineered AAV Rep proteins improved the system's production efficiency and packaging specificity. A comprehensive assessment of the resultant mRNA-carrying AAVs (RAAV) properties was further performed in the liver and brain. Then, our novel RAAVs were applied in gene editing to evaluate the off-target effect. Through multi-step engineering, we achieved a ~6000-fold increase in mRNA loading and a ~14,100-fold reduction in DNA loading with RAAV compared to the conventional AAV. Our

data suggested that these RAAVs retained most properties of conventional AAVs, including capsid composition, virus morphology, and tissue tropism. Importantly, these RAAVs mediated mRNA transfer into the target cells and tissues, leading to transient expression of the functional protein. Our findings also demonstrated that the RAAVs enabled efficient CRISPR gene editing in cells with reduced off-target effects. Furthermore, intravenously injected RAAV with capsid PHP.eB efficiently crossed the BBB and infected the whole mouse brain. We established an efficient, engineered RAAV vector from the DNA virus AAV that specifically and safely delivers mRNAs for therapeutically relevant applications. Our results demonstrate the feasibility of using rational engineering to change the virus genome type. The RAAVs combined the transient nature of mRNA with a variety of tissue tropism of the AAV capsid, making them ideal for either broad-spectrum or tissue-specific mRNA delivery. Our novel approach in developing RAAVs to express DNA transiently will help guide future engineering endeavors to develop safe and innovative delivery systems for *in vivo* gene therapies.

P0167

Effective knockdown of *ATXN2* following intracerebroventricular administration of AVB-205 in BAC-*ATXN2*-Q72 transgenic mice

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Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disorder characterized by the progressive degeneration of motor neurons in the brain and spinal cord, leading to a gradual loss of voluntary muscle control, paralysis, and fatal respiratory failure. Although familial forms of ALS exist (fALS), the multifactorial nature of sporadic ALS (sALS) remains elusive. Recent animal studies have shown that *ATXN2* deficiency disrupts the Ataxin-2-TDP-43 interaction and ameliorates TDP-43 proteinopathy, a causative factor in neurodegeneration in ~97% of ALS and tau-negative frontotemporal dementia (FTD) cases. Thus, *ATXN2* silencing offers a promising therapeutic strategy for sALS and FTD cases. We developed and tested, *in vitro* and *in vivo*, a novel *ATXN2*-targeting miRNA-containing vector from an initial 79 custom-designed miRNA guide sequences. Fifty-one guides with optimal GC content and predicted binding were transfected *in vitro* and 14 sequences were found to knockdown *ATXN2* mRNA (up to 79%). The 14 most promising guides were inserted into our vMiX™ platform, AAV9 vectors produced, transduced in HEK 293T cells, and *ATXN2* mRNA expression quantified. The vector with the greatest *in vitro ATXN2* mRNA knockdown efficacy (AVB-205) was selected for *in vivo* evaluation in BAC-*ATXN2*-Q72 transgenic mice. Four doses of AVB-205, vMiX.CT (Control vector), or vehicle were administered by intracerebroventricular (ICV) injection into neonatal heterozygous BAC-*ATXN2*-Q72 mice and their wild type littermates. After eight weeks, all mice were euthanized and molecular analyses of cortex and spinal cord were conducted to establish *ATXN2*/*Atxn2* mRNA knockdown, vector genomes per cell (VG/cell), and miRNA guide expression. AVB-205 knocked down human *ATXN2* mRNA by 70% in the cortex and 29% in the spinal cord. Mouse *Atxn2* mRNA was knocked down by 59% in the cortex and 24% in the spinal cord in wild-type littermates. In both genotypes and tissues, there was a significant dose-response relationship to knockdown. In addition, a significant relationship between *ATXN2*/*Atxn2* knockdown, VG/cell, and guide miRNA expression was found, such that

an average of 1 VG/cell gave rise to up to 2.8x10^{8} miRNA guides, which repressed *ATXN2*/*Atxn2* expression by 50%. Taken together, this data provides strong support for further testing of AVB-205 in hTDP-43 transgenic mouse models, and its translational potential as a therapy for ALS and TDP43-FTD.

P0168

Novel AAV vectors for improved murine adipose tissue targeting

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Adeno-associated virus (AAV)-based vectors are the leading gene therapy platform and attractive research tools to modulate gene expression in different preclinical models. Various tissues or cell-types of interest can already be transduced efficiently and specifically using naturally occurring AAV serotypes, and engineering AAV capsids can yield novel candidates with improved tropic properties. However, the murine adipose tissue (AT), an important target for preclinical obesity research, remains difficult to transduce, especially after systemic intravenous (i.v.) administration, as benchmark variants AAV8, AAV9, as well as engineered AAV-Rec2 still show strong undesired liver tropism.

Thus, to identify candidates that allow for improved AT transduction in mice, we first investigated the AT tropism of a collection of 34 well-known AAVs, as many of these wild-type and capsid-engineered variants have not been analysed with respect to this target tissue. For parallel screening, we employed state-of-the-art multiplexed biodistribution analyses in C57BL/6 mice using AAV genome-barcoding technology. Hypothesizing that alternative administration routes might reduce liver off-targeting, we not only administered the AAV library i.v., but also intraperitoneally (i.p.), as well as locally into the visceral epididymal white adipose tissue (eWAT). Eight animals per administration route were injected with the barcoded AAV library and DNA and RNA were isolated from up to 18 tissues, including fat depots and off-targets, i.e., liver 14 days later. Vector genomes (DNA) and transcripts (RNA) were then quantified using amplicon-sequencing by NGS.

Local administration by direct eWAT injection exposed novel AAV variants with increased efficiency and specificity for eWAT over liver when compared to benchmark AAVs. For further validation, the AT tropism of these AAVs was individually assessed in a follow-up study via reporter gene expression, revealing AAV-KP1 as highly efficient and specific capsid for local AT transduction. Subsequent functional testing of AAV-KP1 *in vivo* is currently ongoing.
In contrast, while other AAV variants (e.g., AAV7 and AAVrh.10) showed increased AT transduction efficiency after i.p. administration, overall, no variant with improved specificity for AT over liver was identified after systemic i.v. or i.p. injection.

Finally, as the barcoded AAV library screen reaffirmed the lack of a systemically applicable AAV variant for AT targeting, we employed *de novo* capsid engineering to identify novel AAV variants with improved AT transduction over liver. To that end, two AAV9-based random peptide insertion libraries were selected in C57BL/6 mice after i.v. injection and captured from different fat depots (eWAT, inguinal AT and brown AT), followed by subcloning over three iterative rounds. Amplicon-sequencing confirmed successful enrichment of AAV variants in the ATs, and hit candidates are currently selected for a barcode-based validation study.

In summary, we were able to advance the applicability of AAVs for murine adipose tissue targeting by identifying AAV-KP1 as novel vector for local AT transduction and screening of potential new AAV variants for systemic application. Our data enable the targeted investigation of adipose tissue in the context of obesity and possible therapies.

P0169

Generation of heart-specific AAV vectors with cross-species selection for cardiac gene therapy

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Heart failure (HF) remains a leading cause of mortality worldwide, necessitating urgent treatment advancements as there is no curative therapy for those patients. Gene therapy offers great potential for revolutionizing HF care by targeting its genetic or molecular origins. However, successful implementation depends on safe, efficient and selective gene delivery to cardiac cells. Here, engineered adeno-associated viruses (AAVs) present promising vectors due to their heart-targeting specificity and minimized off-target effects. Nevertheless, current available serotypes for cardiac gene therapy such as AAV6 and AAV9 also demonstrate a strong liver tropism, which limits their clinicals use. In fact, there have been numerous reports of fatal liver failure by AAV9-based gene therapy in patients due to the predominant hepatic tropism and still, the serotype continues to be among the vector of choice for treating heart diseases due to its high in vivo myocardial transduction efficiency.

To overcome these limitations, this project aims to develop novel cardiac-specific AAV capsids via an innovative experimental-bioinformatic engineering platform, utilizing mouse and farm pig models. Novel AAV capsid variants with unique transduction properties were discovered in human failing myocardium and in combination with natural heart-targeted known AAV serotypes such as AAV6 and AAV9 were used to generate capsid libraries. Employing DNA shuffling and peptide display techniques, extensive capsid libraries exceeding 1,2*10^7 capsid variants were generated, which demonstrated good producibility and high titres in HEK293 cells. These highly diverse AAV libraries were injected intravenously into the different animal models such as mice as well as pigs and the successful transduced AAVs from the hearts were rescued via PCR. The so-called secondary library contained around 1,8*10^5 variants and was again injected as an AAV library pool. These two selection rounds yielded around 100 cardiac-enriched capsid sequences, which were identified using molecular identifiers via bioinformatic analysis. The double-sequencing strategy by short- (Illumina) and long-read (PacBio, Sequel II) sequencing allowed to further map comprehensive biodistribution in other organs. Subsequent bioinformatical ranking lead to the selection of the top 10 capsid variants displaying highest cardiac enrichment over all other organs, such as liver or kidney and display a unique transduction profile.

In summary, we have established an efficient and adaptive platform for engineering novel AAV capsids tailored specifically for heart-specific gene therapy. In combination with optimized promoter and unique capsids, they hold significant promise for the treatment of both rare and common cardiac diseases that currently lack definitive treatments, marking a significant stride toward transforming the landscape of HF management.

P0170

Leveraging HTS technologies to develop methods for in-depth characterization of rAAV products

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With the success of clinical trials, gene therapy today may represent hope for patients suffering from genetic diseases. Recombinant Adeno-associated virus (rAAV) products are extensively characterised prior to release. Among the Critical Quality Attributes (CQAs) used for release testing, those related to the identity and purity of rAAV products are scrutinized to ensure patient safety and treatment efficacy. Although quantitative polymerase chain reaction (qPCR) PCR and Sanger sequencing are currently among the gold standard techniques, the rapid development of high-throughput sequencing (HTS) technologies has paved the way for a much more exhaustive characterisation of rAAV product's DNA content.

It has been just over ten years since rAAV products began to be closely studied by high-throughput sequencing, initially using Next Generation Sequencing (NGS) with short-reads (e.g., Illumina) and then using Third-generation sequencing, also known as long-read sequencing (e.g., Pacific Biosciences and Oxford Nanopore Technologies). Studies carried out so far show that both short-read and long-read sequencing technologies are of importance for the characterization of DNA, each with its own strengths. Next-generation sequencing offers HTS depth and a low sequencing error rate, enabling exhaustive identification and quantification of contaminating DNA, and detection of mutations at unprecedented levels. On the other hand, long-read sequencing technologies offer the opportunity to sequence DNA at single-molecule level, allowing the identification and quantification of truncation events in rAAV genomes as well as determining the size of contaminating DNA. There is currently no consensus on the method of choice, and a combination of different HTS technologies is needed to obtain the most exhaustive view of the DNA present in rAAV products.

In this study, both Illumina and Oxford Nanopore Technologies were used. We have developed and established all the laboratory protocols, from sample and library preparation to sequencing, as well as the bioinformatics pipelines for providing information on CQAs. To achieve this, experiments were performed to determine the acceptance criteria and guarantee the quality of the results for (1) the payload sequence identity, (2) the payload sequence integrity, and (3) the non-payload sequences characterization. We were able to identify Single Nucleotide Polymorphisms and indels with accuracy; truncation hotspots were detected for the samples used in this study. Also, the presence of contaminants at low levels were detected and quantified.

We believe our methods can be applied to the exhaustive characterization of both DNA starting materials (e.g. plasmids) and rAAV gene therapy products, and, consequently, make it possible to validate HTS-based methods in the near future.

P0171

AAV intein-mediated gene therapy ameliorates dystrophic phenotype in MDC1A mice

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The use of adeno-virus associated (AAVs) as a gene delivery tool has revolutionized the field of gene therapy by providing effective, long-term and targeted treatments for a variety of genetic disorders. In the recent years several treatments using the AAVs have been receiving marketing approval. However, their potential applications are limited by their relatively small cargo capacity (< 4,7 kb). This presents a challenge for monogenic diseases caused by larger genes, such as Merosin-deficient Congenital Muscular dystrophy type 1A (MDC1A) muscular dystrophy, which is caused by mutations in the 9,3kb LAMA2 gene. MDC1A is a monogenic recessive disorder caused by the absence of a functional copy of the Laminin-α2 protein, which is primarily found in the skeletal muscle and Schwan cells. The LAMA2 gene, is a large complex gene with hundreds of different pathogenic variants. Currently, there is no available treatment for MDC1A, and the most promising approach is gene replacement. In here, we have developed a triple AAV gene therapy by splitting the LAMA2 gene in three parts, each vector encoding one of the fragments flanked by short split inteins. Utilizing the natural ability of split intein to perform protein trans-splicing, we achieved full re-constitution of the laminin-α2 protein in vitro and in vivo and we observed an improvement of the histopathological features in the dy^{2j} dystrophic mice model.

P0172

A Novel, Synthetic DNA for efficient rAAV Manufacturing

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The number of FDA-approved gene therapy products using recombinant adeno-associated virus (rAAV) has grown rapidly in the last few years and highlights the key role this vector will play in future gene therapies. However, current manufacturing constraints pose challenges in meeting the increasing demand for rAAV-based therapies. A fundamental challenge is the sourcing of large quantities of plasmid DNA, the essential starting material for conventional rAAV production, which entails long turnaround times and a high probability of alterations in the AAV-specific ITR sequences during the fermentation-based production processes. Furthermore, the plasmid backbone that contain bacterial sequences can potentially be packaged, risking the safety profile of the AAV product.

We have developed a novel, optimized synthetic linear double-stranded DNA with customizable hairpin-ended structures for use in rAAV manufacturing. Our production process has several advantages over that of plasmid DNA since it is a one-pot, enzymatic, cell-free, scalable reaction with high yields and rapid production times. The final DNA product is characterized by superior purity and quality, and better product homogeneity. Interestingly, our customized hairpin-ended structures seamlessly integrate with the function of each synthetic DNA construct. To replicate and package the gene-of-interest (GOI) without any additional unwanted sequence, the hairpin-ended structures of our GOI construct were designed so the full synthetic DNA would mimic a viral genome and would be able of successfully undergoing AAV replication and packaging. In contrast, our synthetic RepCap and Helper constructs are flanked with *de novo* synthetic hairpin-ended structures designed not compatible with AAV replication to prevent undesired packaging of of AAV structural and replication sequences. Furthermore, the absence of bacterial sequences in our DNA material reduces the risk of unwanted DNA sequences in the resulting rAAV product.

Here we show that our synthetic DNA outperformed plasmid construct for single-stranded AAV (ssAAV) or self-complementary AAV (scAAV) manufacturing. Our synthetic DNA resulted in up to 2.5-fold higher AAV titers across a range of serotypes. During scale up to a 400 mL culture, the gain in titer was maintained and even resulted in a 2-fold increase in the fraction of full particles before any downstream processing is carried out. rAAV made with our synthetic DNA exhibited an absence of the backbone sequence observed in plasmid-derived rAAV due to reverse packaging. Importantly, rAAV produced from our synthetic DNA was able to drive eGFP expression in transduced cells and showed comparable infectivity to rAAV produced from plasmid, thus proving its full functionality

Altogether, our results point to our synthetic DNA as a superior alternative for starting material for rAAV production compared to conventional plasmid DNA, stemming not only from the speed and scalability of our production process, but also from the reliability and performance of our final DNA product.

P0173

Developing a Chromatography Method for Purification and Enrichment of Full Capsids in AAV6

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In the development of AAV6 as a gene therapy drug product, empty capsids are formed. These capsids do not contain the gene of interest but can increase the likelihood of immune responses in patients. Therefore, various methods have been employed to remove empty capsids and increase the percentage of full capsids. The most widely used methods are cesium chloride ultracentrifugation, iodixanol gradient ultracentrifugation, and anion exchange chromatography (AEX). Due to the numerous advantages of chromatography over ultracentrifugation in terms of scalability, this approach was selected for further development.

The material for this study was produced in-house, utilizing AAV6 generated in suspension HEK293 cells with a 5.0 kbp oversized client construct as the gene of interest. Post-cell lysis, the material underwent clarification via depth and sterile filtration, followed by concentration and diafiltration through tangential flow filtration (TFF). The TFF retentate was then purified using

affinity chromatography with a novel affinity resin. Success was measured by recovery percentages determined through digital droplet polymerase chain reaction (ddPCR). The elution from the optimized affinity chromatography served as the starting material for anion exchange (AEX) development. Various columns, loading conditions, buffers, and run parameters were evaluated to optimize the enrichment of full particles. Full capsid enrichment was primarily assessed using sedimentation velocity analytical ultracentrifugation (SV-AUC). Promising conditions were further analyzed by ddPCR to determine recovery percentages. The goal of this process is to achieve a high full capsid enrichment of 95% while maximizing product recovery.

P0174

An integrated platform for the analysis and quality control of rAAV vectors based on long-read sequencing

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Recent developments in recombinant adeno-associated virus (rAAV)-based gene therapy have proven its potential and applicability to a broad range of otherwise untreatable diseases. The successes at clinical and pre-clinical level of rAAV-based therapies have quickly elected rAAV vectors as the leading platform for treatment delivery, with multiple therapeutics being already regulatory approved in United States and Europe.

One of the major challenges in proving the identity and quality of the rAAV product is that traditional identity methods such as PCR amplification and DNA sequencing of the encapsulated vector genomes are not sufficient to accurately assess the quality of the material produced. Massive parallel sequencing approaches thus must be adapted to address these specific challenges.

NewBiologix (NBX) has developed an analytical approach for the study and quality control of rAAV vectors specifically targeted to gene-therapy applications. This approach is based on combining long-read sequencing with specialized bioinformatic analyses. Both SMRT-seq (PacBio) and Oxford Nanopore Technologies (ONT) were compared in terms of their sequencing output, read quality, mappability on the vector sequences, percentage of partial and full vector genomes, as well as capability at detecting contaminant plasmid or cellular DNA sequences. NBX also tested multiple strategies for the generation of the second DNA strand for PacBio sequencing.

Results show that, in terms of vector coverage, SMRT-seq can produce a higher proportion of reads spanning the whole ITR-to-ITR sequence with respect to ONT. When assessing the ability to detect DNA contaminants, across our experiments we were able to detect up to 10% of sequences which did not map exclusively to the vector DNA, with comparable results between the two technologies; results for SMRT-seq varied depending on the second-strand generation strategy. In all the experiments, one of the greatest sources of contaminants observed from common transient transfection rAAV production methods are the rep-cap sequences. Such sequences should be avoided as a potential harm and whose source is to be further investigated. These results point towards the necessity of an integrated usage of both technologies for the reliable identification and qualification of the rAAV product.

NewBiologix is actively working on improving the yield and quality of its rAAV sequencing protocol and expanding its computational classification of DNA contaminant sequences.

P0175

Enhancing AAV Purification: Strategies for Improved Recovery and Quality for Downstream Scale Up

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In gene therapy manufacturing, the proper extraction of adeno-associated virus (AAV) material from transfected cell cultures during the upstream process is pivotal for downstream success. However, challenges in AAV manufacturing include limited titer production and difficulties in high recovery of full capsids. This study aims to optimize the initial stages of the downstream process using high-titer AAV material and diverse analytical methods, specifically for AAV8. Notably, we achieved an exceptionally high titer of >1E12 vg/mL post-lysis, surpassing the normative threshold, which serves as our starting material.

High-quality AAV purification is crucial to establish a preliminary downstream purification standard before chromatography. Filtration steps are essential for eliminating residual host cell proteins while preserving viral particles. We conducted a comparative analysis of three vendors, each providing their top three performing depth filters. Following filtration, Tangential Flow Filtration (TFF) was performed as an optimal approach for maximizing recovery and purification.

During these tests, multiple analytical methods, including ddPCR, were used to quantify viral particle counts of AAV. The comparison involved evaluating each filtrate against pre-filtered materials, examining turbidity reduction, reduction in host cell protein and DNA levels, viral particle recovery, aggregation, and considering factors like time and throughput. The selected filtration system, derived from testing 10 different filter trains and supported by advanced analytics, demonstrated >95% recovery, even with a lysate titer of 1E12 vg/mL, generating high-quality AAV materials ready for chromatography. Moreover, we successfully conducted a scale-up study of up to 30 liters using our chosen filtration system, maintaining the same high yield. Data from viral titer recovery using ddPCR and assays gauging residual host cell proteins provide valuable insights for optimizing AAV purification processes. This research enhances efficiency and quality in AAV production, advancing gene therapy applications.

P0176

Novel generic digital PCR-based method to detect and quantify replication-competent AAV genomes in AAV vector-based products

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Replication-competent AAVs (rcAAV) are gene therapy product-related impurities. They are composed of a viral vector capsid containing wild-type AAV-like genomes including the rep and cap genes with functional promoters, allowing their replication and generation of infectious particles in the presence of a helper virus. While wild-type AAV has no known associated pathology, there could be immunogenicity or genome integration risks associated with the unintended replication of AAV-like particles, should a helper virus co-infect the target tissue of the AAV vector-based gene therapy.

The standard rcAAV detection method is a cell-based assay consisting of three successive rounds of amplification in a permissive cell line in the presence of a helper adenovirus. After the three rounds of amplification, the presence of Rep2 and Cap gene sequences is quantified by qPCR to establish the presence of rcAAV. In terms of gene therapy product release testing, the absence of rcAAV detection in 1E+08 vg product dose equivalent has generally been used as an acceptance criterion for this specification.

However, significant method differences between testing facilities, including but not limited to the choice of the testing permissive cell line, could lead to major variations in rcAAV detection and relative quantification across AAV vector-based gene therapy products.

A novel method has been developed that uses a multiplex digital PCR (dPCR) for the detection and quantification of putative rcAAV genome in rAAV products. This triplex dPCR method is designed to detect 3 junctions specific to rcAAV genome: ITR-Rep, ITR-Cap and Rep-Cap. rAAV particles containing the 3 targeted junction sequences ('triple positive') are considered complete rcAAV particles, whereas particles containing 1 or 2 targeted junctions out of 3 represent partial genomes and thus are not considered replication competent. Analysis of several rAAV batches of serotypes Cap2 and Cap8 with this novel assay showed similar levels of triple positive genomes ranging from 0.000007 to 0.00008 % relative to total vg copies. In contrast and unlike AAV2/2, no rcAAV2/8 was detected in the cell-based assay, likely due to the discrepancy in detection sensitivity between the 2 serotypes in this method. Indeed, it was observed that of 10 IU of wild-type-like AAV preparations as measured by TCID50 corresponded 50 and 50,000 triple positive genomes for AAV2/2 and AAV2/8, respectively. Therefore, it appears that the cell-based assay results in an underestimation of the potential for rcAAV in AAV2/8 preparations. In addition, this assay does not reflect the higher infectivity of AAV2/8 vectors in vivo in multiple target tissues compared to that of AAV2/2.

The novel triplex dPCR assay offers a more sensitive and unbiased analytical method for the detection and quantification of rcAAV genomes in AAV-based vector preparations that could be applied to process development and release testing purposes in gene therapy.

P0177

Gene therapy risk assessment: viral vector integration into the host genome

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The integration of viral-derived vectors and wild-type viruses into unintended genomic regions can have severe consequences, including tumorigenesis, insertional mutagenesis, and viral evolution within the host genome. These off-target and adverse events pose significant biosafety concerns, especially in the context of cell and gene therapy. However, current methodologies for detecting viral integrations, particularly those occurring at low frequency, are limited.

Recent advancements in target enrichment sequencing and the development of accurate computational detection tools have created new opportunities for biosafety assessment. We have thus implemented an experimental and computational pipeline able to detect viral integrations with high precision. This pipeline leverages the VSeq-toolkit, a state-of-the-art viral integration detection tool, on targeted viral-host genomic regions, enhancing the sensitivity and specificity of integration detection. We have optimized the experimental protocol, testing different library preparation kits and capture panels for cost-effectiveness and reducing labour intensity.

To validate our computational approach, we conducted comprehensive evaluations using 18 simulated datasets that varied in the number of integrations, integration patterns, viral vector types, read lengths, and other sequencing and molecular complexities. Our results demonstrated that the pipeline performs robustly across most scenarios, detecting > 95% of integrations with no false positives for typical target enrichment coverages.

We successfully applied our pipeline to investigate therapeutic vector integrations, such as *AAVLK03OTC*, and natural integrations of *HIV-1*. Additionally, we explored natural integrations of *AAV2* and *HHV6* in children with acute hepatitis of unknown origin. These studies provided valuable insights into the patterns and behaviours of viral integrations, aiding in the understanding of potential adverse events.

To support scientists and clinicians, we aim to create a user-friendly reporting format for our integration analysis. This format includes detailed information on the frequency and distribution of viral/vector integration sites across different genomic regions, including both genic and intergenic. This comprehensive analysis will be offered as part of a future service aimed at assessing genomic risks in therapeutic contexts at UCL Genomics.

This service will enhance biosafety monitoring and support the development of safer gene therapies by providing precise and actionable insights into viral integration events. Our pipeline's ability to detect integrations accurately, even at low frequencies, represents a significant advancement in the field and underscores the importance of integrating experimental and computational methodologies for comprehensive biosafety assessments.

P0178

Screening Assays for Anti-AAVrh.10 Antibodies in Support of Gene Therapies: TAb or NAb?

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Immunogenicity assessments as inclusion/exclusion criteria to predict investigative drug safety and efficacy are critical in the development of Adeno-Associated Virus (AAV)-based gene therapies. Although multiple assay formats which measure distinct endpoints (i.e., binding versus neutralizing antibody activity) are applicable and are already being used in this context, it is not

clear which assays should be selected and at what stage of drug development these methods should be implemented. AAVrh.10 has gained interest as a gene therapy platform due to its superior transduction efficiency, however there is limited information in the literature on pre-existing anti-AAVrh.10 antibodies and their clinical impact. In this presentation, we highlight the validation of a total antibody assay (TAb) and a neutralizing antibody (NAb) assay for detection of anti-AAVrh.10 antibodies for enrollment of patients into an AAVrh.10 gene therapy clinical study. The AAVrh.10 TAb and NAb validation data sets are compared in terms of assay sensitivity, drug tolerance, and precision, along with a concordance analysis to determine if the assays produce similar results when used for screening a population of potential patients. This comparison will give insight into the utility of each assay as a potential tool for inclusion of select individuals into clinical studies.

P0179

Optimization of viral protein ratios for AAV5 production in insect cells

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The baculovirus expression in *Spodoptera frugiperda* insect cells (Bac/Sf9) system, known for its scalability, suspension culture capability, high biosafety, and efficient transfection, offers a versatile platform for adeno associated virus (AAV) vector production. However, AAV intron splicing signals are poorly recognized in Sf9 cells, leading to reduced activity of AAV vectors produced in insect cells mainly due to suboptimal VP1 protein expression. The N-terminal region of VP1 protein features a unique Phospholipase A2 domain absent in VP2 and VP3. This domain is vital for transduction of AAV, facilitating endosome exit and viral genome transfer into the nucleus. For some AAV serotypes a correct VP protein ratio can be easily achieved by eliminating splicing signals and using alternative translation initiation codons (like CTG or ACG) for VP1 translation. However, modification of the initiation codon alone does not work for all serotypes.

Here we describe an efficient modification of Rep2Cap5 construct for use in the Bac/Sf9 system. To create AAV5 VP protein expression cassette we utilized baculovirus P10 promoter, small intron derived from the Drosophila myosin heavy chain gene and, combination of translational enhancers and attenuated Kozak sequences. This set of regulatory sequences enabled us to achieve high AAV5 VP1, VP2 and VP3 expression at ratio very close to 1:1:10. Using optimized AAV5 VP protein cassette in vector production we obtained high AAV5 yields (up to 2.44×10^{14} vg/L) and infectivity in our Bac/Sf9 platform that is comparable to AAV5 vector produced in the HEK293 cells. We also demonstrated that the same expression regulation system could be used for Rep78/Rep52 protein expression in proper ratio. The developed AAV5 production system is suitable for incorporation into scalable platform of GMP-grade vector production system.

P0180

Modification of Adenoviral helper and RepCap plasmids to improve adeno-associated virus (AAV) yield and safety

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Recombinant adeno-associated virus (rAAV) is commonly generated by using triple plasmid transfection including Adenoviral (Ad) helper, RepCap, and GOI plasmids into HEK293 cells. It has been widely reported that a minimum of five Adenoviral (Ad) elements (E1a, E1b, E2a, E4ORF6, and VA RNA) are required for production of AAV. During optimization of Ad helper plasmid, we identified a sixth region encoding for the Ad L4 22/33K proteins that was previously not recognized as an essential region for AAV production. We found Ad helper plasmid variants that lacked L4 22K protein expression could not support AAV production, but that AAV yields could be restored if the L4 22K gene was supplied in trans. We also demonstrated that expression of L4 33K protein synergistically increases rAAV yield and may boost rAAV production if overexpressed transiently. We removed all extraneous Ad genes and created a series of Ad helper plasmids with reduced size (7 to 9 kb) to improve plasmid manufacturability. The minimized Ad helper plasmids were tested in larger scale production. Modified Ad helper plasmids successfully performed in both single-stranded and self-complementary rAAV production, using various AAV serotypes and vectors transgenes. Addition and modification of certain genetic elements in the smaller Ad helper plasmids led to significant increases in rAAV yield.

We have also further improved the RepCap expression plasmid by gene shuffling, altering codon sequence, and site-directed modification of the RepCap plasmids. The substantial rAAV yield increases were achieved for several serotypes including AAV2, AAV6, AAV9, AAVrh10 and AAVrh74. Combination of minimized Ad helper plasmid and modified RepCap plasmids led to additional rAAV yield increase by 2-5 fold depending on AAV serotype.

Systematic evaluation and modification of genetic elements of rAAV production plasmids empowered higher production yields, facilitating large-scale manufacturing necessary for commercial and clinical use. Removal of non-essential genes and genetic elements from Ad helper plasmid improves safety features and minimizes potential hazards of adverse reactions for gene therapy recipients.

P0181

Engineering Cell Type Specific Adeno-Associated Virus (AAV) by Computational Protein Design

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Vectors derived from Adeno-Associated Viruses (AAVs) have enabled many gene therapy applications by leveraging virus natural ability to introduce genetic material into cells. However, their broad tropism and limited specificity to cell types, tissues, and organs can lead to adverse

side effects caused by liver toxicity and genome transfer in non-target tissues. Here, we report an AAV retargeting platform to address this limitation by functionalizing AAV capsids with computationally designed miniproteins binding to cell-type specific surface receptors. Our approach is based on the hypothesis that such *de novo* designed miniproteins are suited to re-target natural AAVs whose primary-cell attachment has been depleted.

Traditionally, the discovery of binding proteins involves the screening of large randomized libraries with little or no control over the resulting binding site and interaction mode. Utilizing state-of-the-art protein structure prediction networks, we developed a computational design pipeline to generate relatively small (<150 amino acids) binding proteins with high experimental success rates. Our benchmark against several targets allowed us to obtain experimentally validated binding proteins by testing a pool of only 10-20 designs per target. Encouraged by these results, we tailored this pipeline to enforce a minimal distance between the termini of the designed proteins, to allow for effective integration into specific loops at the surface of the capsid and screening of candidates in the context of AAVs.

As a proof-of-concept for vector re-targeting, we have initially inserted a PD-L1 binding miniprotein into the AAV capsid. The capsid was first engineered by mutating key glycan-interacting residues to deplete the primary cell surface attachment of AAV6 and generate a knockout ('KO') transduction-deficient vector. To test our approach, the 'KO' vector was next modified by genetic insertion of the PD-L1-binding miniprotein into the capsid. Chimeric vectors were effectively produced in HEK293 cells, as the yield of genome-containing particles was similar to unmodified AAV6. Mass spectrometry confirmed the presence of the miniprotein on the vector. Further analyses showed that the capsid stability remained unaffected by the miniprotein insertion. *In vitro* transduction assays with these variants demonstrated a selective recovery of infectivity on cells overexpressing the respective target receptor PD-L1.

We are currently testing the possibility to generalize our approach to other target proteins, leveraging efficient *in silico* binder design tools. Using this approach, we aim to expand our retargeting approach to several cell surface receptors to showcase the potential of computationally designed proteins for gene therapy applications. The selected cell surface receptors are validated markers for oncological malignancies and could open a full realm of applications for the delivery of cancer sensitizing genes to make tumors more prone to first-line therapeutics. Furthermore, we intend to characterize promising variants biophysically and assess their performance in tumor-killing assays. This work will lay the foundation for evaluating *in vivo* efficacy of this approach in future studies.

P0182

Optimising Adeno-Associated Virus Delivery Routes for Efficient Gene Therapy Targeting the Central Nervous System

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The utilisation of viral vectors in gene therapy has become increasingly prevalent due to their high efficiency and safety in delivering genetic material. Viral vectors, particularly adeno-associated viruses (AAVs), are suitable vectors for gene therapy, facilitating the targeted delivery of therapeutic genes to specific cells or tissues while maintaining low immunogenicity. However, off-target effects can occur, potentially leading to unintended consequences and limiting therapeutic efficacy. Therefore, careful consideration and optimisation of viral vector selection, serotype specificity, and delivery methods are essential for the success of gene therapy.

AAVs are small, single-stranded DNA vectors that exhibit various serotypes, each capable of targeting specific groups of cells. The choice of AAV serotype significantly influences the efficiency of gene therapy delivery.

Targeting the central nervous system (CNS) with gene therapies presents a formidable challenge, not only due to its inaccessibility but also due to the blood-brain barrier (BBB). Nevertheless, specific serotypes such as AAV9, AAVrh10, AAV-PHP.B, and AAV-PHP.eB can traverse the BBB and reach the CNS. The ability of AAV9 to cross this barrier permits efficient gene delivery to the CNS, making it a powerful tool for treating neurological disorders without the need for invasive procedures.

In this study, we investigated the biodistribution of AAV9-GFP by delivering viral particles via different administration routes: intracerebroventricular (ICV), intraperitoneal (IP), and facial vein injection. The objective was to determine the optimal administration route based on delivery efficiency, invasiveness, complexity, and the vector's capacity to cross the BBB. Newborn mice, one day old, were injected with AAV_CMV_GFP at a dose of 1.3×10^{13} vg/kg. All injections were performed manually using ice anaesthesia. For ICV injections, we used the mouse eye and the lambda on top of its head as references.

Four weeks post-injection, brain, lung, heart, and liver tissues were collected for immunohistochemistry (IHC), biochemical staining, and molecular analysis. Techniques employed included IHC, quantitative PCR (qPCR), haematoxylin and eosin (HE) staining, Western blotting, and direct fluorescence microscopy. These methods enabled us to quantify the distribution and expression of the delivered gene, evaluate tissue integrity, and assess the overall success of gene delivery.

Through the direct administration of AAV9-GFP into the CNS via intracerebroventricular injection, we observed widespread distribution of the GFP protein throughout the brain, including the frontal cortex and deeper regions such as the pons, hypothalamus, and midbrain. Additionally, GFP expression was observed in various other body organs, including the lungs.

Using IP injection, the least invasive administration route tested, we found that AAV9-GFP was able to cross the BBB and achieve more homogeneous expression across the brain. Robust expression was observed in the cerebral cortex, olfactory bulb, hippocampus, putamen, anterior olfactory nucleus, ventral striatum, and cerebellum. This indicated that administration, even when not directly to the brain, allowed for more homogeneous distribution within the organ, as it naturally entered through the blood vessels. Nevertheless, due to the greater distance from the target area, a significantly higher number of viral particles were required, accounting for losses that could occur before reaching the brain.

P0183

Infectivity measurements of AAV vectors using TESSA-Rep Enabled AAV titration (TREAT)

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Measuring the infectivity of recombinant adeno-associated virus (AAV) vectors is a key bottleneck impacting their use as a gene therapy, highlighting the essential need for the development of sensitive and robust analytical assays. The use of wild-type AAVs or engineered cell lines encoding the AAV Rep and Cap genes (e.g. HeLaRC32), combined with wild-type adenoviruses (Ad) to supply the necessary components for recombinant AAV genome replication is commonly employed for infectious titration of AAV. However, high assay variability and safety concerns associated with the use of wild-type viruses can limit their adoption in non-specialized laboratories. Here we developed a novel AAV infectious assay 'TESSA-Rep Enabled AAV titration' (TREAT) based on our Self-Silencing Adenovirus' (TESSA) platform. TREAT employs a self-repressing TESSA virus that expresses all adenoviral helper genes alongside AAV Rep to enable DNA amplification of the AAV within infected cells for quantification via the Tissue Culture Infectious Dose (TCID50) assay. Using a TESSA virus expressing AAV Rep, we showed robust amplification of the rAAV genome (~1x10^4 fold) to enable detection (via qPCR) from a single AAV infection event. The TREAT assay enables the titration of various serotypes of AAV in a wide range of target cell types and provides a more informative infectious titer for research and clinical applications.

P0184

AAV capsid optimization for direct intramyocardial gene delivery

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Direct intramyocardial gene delivery is a promising method for localized gene delivery to the heart, while also showing potential for transduction of a large area of myocardium using multiple injections. Direct intramyocardial injection represents the primary delivery route for local applications such as for example cardiac regeneration and treatment of malignant ventricular arrhythmias. AAV serotypes AAV6 and AAV9 have thus far been most effective in this setting, although still requiring relatively high doses to obtain significant transduction. Moreover, previously published efforts have largely focused on optimizing myocardial transduction following intravenous vector administration, while no dedicated effort has been reported on improving transduction in the setting of direct intramyocardial injection. To improve AAV-mediated transduction following direct injection of myocardium, random peptide insertion libraries were generated by insertion of a 7mer peptide in VR-VIII of AAV6. A complex library was pre-screened in neonatal rat ventricular myocytes. Top performers were generated as a barcoded CMV-eYFP

reporter library and injected intramyocardially in the apex of female FVB mice. Again, top performers were chosen and generated as single non-barcoded cardiomyocyte specific cTnT-GFP and CMV-GFP vectors and injected intramyocardially in the apex of FVB and C57/Bl6 mice. In all cases, four weeks post-injection, mice were killed and the apex of the heart, a remote part of the left ventricle and several off-target organs were isolated. RNA from isolated organs was extracted and analyzed using either next-generation sequencing of barcodes or qPCR on RNA. Selections in neonatal rat ventricular myocytes yielded a small set of highly enriched capsids. 40 top enriched novel AAV6 variants were then included in the barcoded library, together with WT AAV 1, 2, 3, 5, 6, 8 and 9, AAV-DJ, and 9 previously published peptide insertion variants based on AAV2 and AAV9. *In vivo* validation revealed significantly higher RNA expression from AAV-DJ and three novel AAV6 variants compared to wild-type AAV6 in the injection site. Additionally, heart-to-liver ratios were higher for the novel AAV6 variants. Single vector injection using cTnT-GFP vectors showed that our novel AAV6 vector variant (designated AAV6-1) led to a 20x higher GFP fluorescence signal than AAV6-WT, while AAV-DJ led to a 5x higher GFP fluorescence signal than AAV6-WT. In conclusion, we identified a novel AAV6 variant which leads to up to 20x higher transgene expression compared to AAV6-WT, while maintaining a high level of cardiomyocytes specificity.

P0185

The role of thermal stability in AAV titration of engineered variants

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Determining accurate and reproducible titers for recombinant adeno-associated virus (rAAV) productions is essential to their development and downstream therapeutic applications. Lately, a number of engineered serotypes have shown expression in cells that are poorly infected by traditional variants. However, several of these serotypes have low titers, questioning their adequacy for clinical development and use.

We thus decided to investigate how reproducible and reliable their titers were as titering methods are a known bottleneck in the field of AAV-mediated gene therapy. We hypothesized that the DNAse digestion step applied prior to amplifying viral genomes through qPCR or ddPCR, in order to eliminate the free-floating DNA, might constitute a bias : DNAse digestion usually ends by an inactivation step of the enzyme, at 65°C or above, which may adversely impact capsid stability.

We measured melting temperatures (Tm) for selected engineered AAV variants and demonstrated a correlation between lower thermal stability and lower titers with greater variability. Our results show, for the first time, that inactivating rAAV without heating yields significantly higher titers for variants with Tm close to the DNAse inactivation temperature, while titers for parental serotypes with higher Tm remain unchanged.

This finding is important for work involving engineered variants with lower thermostability, and especially when comparing their effectiveness to their parental serotypes.

P0186

Design of experiments in rAAV manufacturing: Optimization of plasmid ratio for better upstream yield

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Recombinant adeno-associated viruses (rAAV) are very popular for gene delivery by virtue of their tissue specificity, low pathogenicity, and long-term efficacy. Since the FDA approval of Luxturna in 2017, six more AAV-based therapies have entered the global market including Beqvez™ for the treatment of haemophilia B. The one-time treatment for severe haemophilia B either with Hemgenix® or Beqvez™ has a price tag in the US of $3.5 million per dose. One of the most expensive raw materials for making rAAVs by transient transfection are the plasmids, that code for the different parts of the virus including the outer capsid. Identifying the optimal amounts of plasmids needed to produce the most, functional rAAVs – albeit from a seemingly endless number of possible combinations – is key to driving down the cost of these therapies. Design of experiments (DoE) is a statistical approach to identify how multiple factors affect a response, and reduce the number of conditions, while still maintaining statistical power. To optimize the production of rAAVs, we reduced the number of 3-plasmid ratio combinations needed to be tested from 14553 to 28, by generating an optimal design using an R-based open-source DoE pipeline. A HEK293F-derived clonal suspension cell line was transfected using the 3-plasmid system in vented 50 mL tubes with 10 mL of cell culture. Cells were harvested 70 hours later by surfactant lysis and endonuclease digestion then clarified by centrifugation. The clarified cell lysate was measured by ITR2-qPCR. We identified conditions of total plasmid DNA along with the optimal ratio of pHelper to pRepCap to pTransgene which had up to 4x higher productivity than others. The results were used to create a Response Surface Model (RSM), which was confirmed by testing the values suggested by ridge analysis, followed by a comparison of our previously best-performing plasmid ratio against the ratio suggested by the RSM. An increase of up to 3x more vector genome titres across four common serotypes and two transgenes of different lengths was found, with productivity for some serotypes at >7E+14 VG/L of clarified cell culture. The method can be applied at the pre-discovery stage to identify capsids and transgenes with better productivity, or later during the preclinical stage to fine tune the plasmid amount for better empty:full ratios.

P0187

AAV vector glycoengineering effects on transduction

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Adeno-associated viruses (AAVs) are non-glycosylated but they use glycans like heparan sulfate and/or galactose residues on cell surfaces as co-receptors for cellular transduction. As most enveloped viruses are heavily glycosylated we were interested in exploring the effect of AAV9 surface glycosylation to modulate vector tropism and/or improve vector transduction. To this

end, we have prepared a collection of structurally varied synthetic glycans for attachment to the capsid and studied the influence of glycan structure, attachment site, and glycan density on vector internalization and cell transduction both *in culture* and *in vivo*. We have linked glycans to three different residues near the AAVR interaction surface and found that the cell transduction capacity of the modified vectors, both *in culture* and *in vivo*, is strongly dependent on glycan capsid density and structure. Generally, a high density of glycans negatively affects AAV transduction, but this effect is influenced by the glycan structure, which alters the degree of modification that reduces AAV transduction. Additionally, sialylation of certain glycans restores AAV infectivity *in vivo*.

Glycoengineering offers an opportunity to provide new properties to AAVs. However, this vector modification must be approached cautiously, as certain limitations related to glycan density and structure need to be considered to avoid affecting AAV infectivity.

P0188

Improving quality & economics of AAV Manufacture with dbDNA™

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The increasing success of AAV gene therapy for large indications results in greater pressure on manufacturing to produce high quality, high titre AAV batches. For mass production of adeno-associated viral (AAV) vectors, striving to improve multiple parameters can impact the cost of manufacturing. The goal of these improvements is to find those parameters that not only improve the final drug product but do so in a more cost effective manner. Touchlight's doggybone™ DNA (dbDNA™) is an enzymatically made, linear, closed-ended, double-stranded DNA that can be used as an alternative to plasmid DNA (pDNA), in many therapeutic modalities including mRNA, genome editing, cell therapies, DNA vaccines, and viral vectors. In the latter, it can be used to drive down costs of goods per batch and improve yields and quality of the final gene therapy product.

Evaluations of AAV production parameters were performed comparing dbDNA and pDNA. The use of dbDNA for production of AAV vectors, in both shake flasks and bioreactors, has been shown across multiple projects to require less DNA input averaging 40% lower mass and transfection reagents quantities are reduced up to 50%, compared to pDNA. Importantly, utilisation of dbDNA for AAV manufacture has produced equivalent or greater titres of viral genomes (VG), a decrease in viral particles (VP), and an increase in encapsidation of those genomes. These experiments demonstrated an impressive 2-fold improvement on average to the proportion of full capsids compared to pDNA-manufactured AAV, confirmed by mass photometry after simple AEX chromatography process.

These improvements in upstream production have led to improved downstream process recovery amounts and the ensuing polishing steps with final vector product results generating greater than 80% full capsids as measured by mass photometry. Next generation sequencing of AAVs produced by dbDNA have shown decreased impurities when compared to AAV produced using plasmid DNA. These data provide support that dbDNA also offers improvements to the quality of AAV.

In summary, switching to dbDNA for the manufacture of AAV results in increased full capsids and also offers improvement to the impurity profile of the AAV particles. This, coupled to the reduced amounts of DNA and possibly transfection reagent, leads to an overall cost of goods decrease when implementing the use of Touchlight's dbDNA.

P0189

Advancing clinical trial readiness to expedite the development of therapies for CTNNB1 neurodevelopmental syndrome

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CTNNB1 syndrome is a rare autosomal dominant neurodevelopmental disorder diagnosed in approximately 500 patients worldwide. Mutations linked to this syndrome are found in the CTNNB1 gene, which encodes β-catenin, a crucial component in Wnt signalling. Genetic therapies for CTNNB1 are in early development, with AAV9 vector gene replacement showing promising results in cell lines, organoids, and mouse models. These studies demonstrate effective β-catenin replacement and phenotype improvement, with favourable biodistribution and safety profiles. To expedite therapy development for CTNNB1 patients, it is crucial to fully characterize the syndrome's phenotypes, natural history progression, and identify biomarkers for valid clinical trial endpoints. Here, we present a genotype-phenotype analysis and preliminary results from the natural history study.

A genotype-phenotype correlation study conducted in 2021-2022 included 127 CTNNB1 patients to understand the link between genetics and symptoms. The study examined gene expression and the tendency for nonsense-mediated mRNA decay. Consistent dysmorphic features were observed, with central hypotonia, peripheral hypertonia, dystonia, and spasticity being the most common neurological symptoms. Motor developmental milestones were significantly delayed, and about 25% of patients experienced regression in speech or independent walking. Intellectual disability ranged from mild to severe. Most mutations are predicted to be loss-of-function, though rare cases may exhibit dominant-negative or gain-of-function effects.

In June 2024, 83 children with a confirmed genetic diagnosis of CTNNB1 syndrome participated in a prospective, longitudinal, observational natural history study. A total of 249 participants, including individuals with CTNNB1 syndrome and their careers was included. The study involves annual visits over five years for data collection through clinical examinations, interviews, validated questionnaires, and carer diaries. Blood samples for genetic and serum analysis, electrophysiological recordings, neuroimaging for structural abnormalities, and actimetry for gait/movement analysis using an ActiMyo/Syde® CE-approved device were collected. Preliminary data from this study will be presented, offering valuable insights into the natural progression of CTNNB1 syndrome and aiding in the development of effective therapies.

P0190

Integration of protein language models and reinforcement learning for the systematic design of phenotype-specific AAV capsids

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Effective gene therapy development hinges on the precise engineering of AAV (Adeno-Associated Virus) capsids that exhibit tailored phenotypes enhancing therapeutic efficacy and safety. This study presents a computational strategy that combines protein Language Models (pLMs) and Reinforcement Learning (RL) to generate novel amino acid sequences for AAV capsids, targeting specific phenotypic traits. The model proposes new sequences made by amino acid permutations in the constitutive variable region (VR VIII). This approach will lead to future developments addressing critical challenges in gene therapy such as immune evasion, tissue-specific targeting, and transduction efficiency.

A Protein Language Model (ESM-2) has been employed to accurately encode amino acid sequences based on comprehensive protein functional and structural data. The study aimed to train a model encoding amino acid sequences to generate novel manufacturable AAV variants (viability). Reinforcement Learning is subsequently used to iteratively refine these sequences towards optimal phenotypic performance, adhering to predefined structural criteria. This dual approach facilitates the development of AAV capsids that are sampled in a larger space than the initial one.

The RL rewards are based on a previously trained viability classifier on independent data, with an F1-score of 0.95, Recall of 0.95, and Precision of 0.94.

Then the model has been trained to learn a policy over 4 possible actions (deletion, addition, substitution and inversion) with a custom reward function if the generated sequence turns out to be viable according to the model above.

Preliminary results from the research indicate that modifications designed through this AI approach might lead to maintaining or enhancing the capsids' intended phenotypic characteristics, to be validated experimentally.

This study highlights the benefit of combining advanced computational techniques to assist in the intricate task of designing functional gene therapy vectors, thereby enhancing the precision and efficiency of therapeutic interventions.

P0191

Impact of cell line selection on AAV2 viral vector genome packaging efficiency

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Viral vectors derived from Adeno-associated virus (AAV) are being used to introduce a variety of gene therapies into humans at an ever-growing rate. A vast amount of effort has been expended to improve the manufacturing and delivery of AAV vectors, in terms of packaging efficiency, targeted delivery and minimization of off-target effects, amongst others. However, one aspect of AAV production in need of further optimization is increasing the proportion of AAV vector particles that contain full-length genomes, rather than truncated DNA or empty capsids.

CTx001 is an AAV2 gene therapy encoding a novel soluble and potent complement regulatory protein, mini-CR1, for the treatment of Geographic Atrophy. Mini-CR1 is derived from the membrane bound complement receptor 1 (CR1) protein. Two different cell lines were assessed to manufacture CTx001: the conventional HEK293 cells adapted to suspension, and the VPC2.0 from Thermo Fisher. A significantly different percentage of partially filled capsid was observed in CTx001 vectors produced in the two cell lines. To further characterize this difference, a suite of analytical assays was used. These assays included mass photometry, analytical ultracentrifugation (AUC), long read sequencing analysis, alkaline gel electrophoresis and a ddPCR assay that targeted different regions of the genome. Whilst CTx001 produced in the VPC2.0 cell line displayed the expected profile of full and empty capsids, CTx001 generated in the conventional HEK293 cells had a very significant proportion of truncated DNA.

In summary, changing from conventional HEK293 cells to VPC 2.0 resulted in a dramatic reduction in the amount of AAV2.CTx001 vector particles containing truncated DNA. This reduction was corroborated using a number of analytical techniques. These results demonstrate the substantial impact the choice of cell line can have on AAV viral vector genome packaging efficiency.

P0192

Development of Potency Assays for AAV Encoded Transgenes Regulated by Photoreceptor and Retina Specific Promoters

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1: Svar Life Sciences

An essential safety requirement for the successful development of therapies based on AAV-encoded transgenes is that the transgene is expressed exclusively in the target tissue. This can be accomplished by the use of tissue or even cell-type specific promoters. This poses significant challenges, however, for the development of appropriate potency assays that accurately reflect

tissue or cell-type specific expression of the transgene. The i*Lite*® reporter-gene technology has been used to develop potency assays for AAV encoded transgenes regulated by both photoreceptor-specific promoters and retina-specific promoters that reflect to a high degree the mechanism of action of the AAV-encoded transgene. The development of one such assay for the quantification of the potency of the retina-specific guanylate cyclase GUCY2D regulated by the hGRK1 promoter derived from the rhodopsin kinase G-protein coupled receptor kinase will be described. The assay is based on the stable co-transfection of ARPE-19-HPV-16 cells, derived from the retina pigmented epithelium, with a highly specific cGMP responsive reporter-gene construct, that does not respond to cAMP, and guanylyl cyclase-activating protein-1 (GCAP-1) to obtain maximal GUCY2D activity. The resulting cell line was also stably transfected with the Renilla luciferase (RL) reporter-gene under the control of a constitutive promoter that allows both FL and RL activity to be quantified sequentially in the same well of a microtiter plate. FL activity can thus be normalized with respect to RL expression rendering the assay independent of cell number and allowing non-specific effects to be detected. The resulting i*Lite*® cell line has been used to quantify the potency of an AAV5 encoded GUCY2D transgene under the control of the hGRK1 promoter. A dynamic range of some 750-fold was obtained in a 4PL potency assay and a relative potency assay without the need to add live Adeno virus or other external stimuli. The cells have been converted into a frozen ready-to-use format that confers both ease of use and low coefficients of variation. Data will be presented illustrating the utility of this assay.

P0193

Investigating rAAV drug product stability at different temperatures and primary packaging

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As more rAAV-based gene therapy products reach the market, the promise of these therapeutics to tackle diseases that were previously out of reach comes closer to becoming a reality. Unfortunately, along with that comes the realisation that the stability of these viral vectors is really limited when it comes to non-frozen conditions. With the current trend of extending the reach of gene therapies out of the rare disease space, the need for stable formulations becomes even more urgent.

We have previously presented our investigations on formulation parameters, such as pH, salt, and sugar content, which can impact the stability of commonly used rAAV serotypes under thermal stress. In our quest to generate a stable rAAV ‘drug product’, we have now expanded our exploration to a selection of realistic storage temperatures, apart from accelerated degradation at higher temperatures. Additionally, standard primary packaging for rAAV ‘drug product’ has been taken into account for the different temperatures, since the stability of the formulation and especially titer loss due to adsorption, can be affected by the material of the primary packaging. To that end, emphasis was placed on the importance of the surfactant type and concentration selection to prevent adsorption while not adversely affecting the activity of the viral vector.

Together, our results paint a picture of the effect of formulation, temperature, and primary packaging on rAAV stability and could point towards formulation parameters that could prolong the shelf life of rAAV formulations at higher temperatures.

P0194

Determination of full-empty ratio of AAV Standard Material by Orthogonal Methods

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Today, AAV-based gene therapy is one of the most promising and fastest growing branches of modern medicine. Moving forward to a more clinical focus in this field, the use of reliable AAV standards and orthogonal methods is indispensable to meet current FDA guidelines. Since the production of comprehensively characterized, internal standard material is not feasible for a large number of companies and a lack of commercially available reference material, we now offer AAV standard material for the most commonly used AAV serotypes. We have established a comprehensive characterization process for these commercially available standards. Besides testing several characterization parameters (e.g. vg and capsid titer, filling grade, purity and aggregation), this process also contains the analysis with a set of suitable orthogonal methods ensuring the utmost level of accuracy and reliability of the data.

Here, we present our study on current orthogonal methods for determining the full-empty ratio and their comparability using our eGFP-filled AAV standard material (AAV2, AAV5, AAV8 and AAV9). The study includes the full-empty analysis using Charge Detection Mass Spectrometry (CD-MS), Size Exclusion Chromatography – Multi-angle Light Scattering (SEC-MALS), Mass Photometry (MP), and a combination of Dynamic Light Scattering/Static Light Scattering and Ultraviolet and Visible Light Measurements (DLS/SLS-UV/vis).

Depending on the serotype and the method, some variations were observed regarding the filling grades. The average filling grades across serotypes were measured by DLS/SLS-UV/Vis, CDMS, SEC-MALS, and MP. The results obtained from CDMS and MP showed in average the highest correlation, while a little higher and lower full-empty ratios were observed using SEC-MALS and DLS/SLS-UV/Vis, respectively.

The use of reliable standards within diverse orthogonal methods ensures not only the most accurate result but also the safety of the final product in compliance with current FDA requirements. Therefore, we present the reliability of our characterization process for AAV standards, thus demonstrating their reliability and suitability for biophysical and immunological applications, respectively.

P0195

AAV Capsid Titer Determination of AAV Standard Material by Orthogonal Methods

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Today, AAV-based gene therapy is one of the most promising and fastest growing branches of modern medicine. Moving forward to a more clinical focus in this field, the use of reliable AAV standards and orthogonal methods is indispensable to meet current regulatory guidelines. Since the production of comprehensively characterized, internal standard material is not feasible for many companies and a lack of commercially available reference material, we now offer AAV standard material for the most used AAV serotypes. We have established a comprehensive characterization process for these commercially available standards. Besides testing several characterization parameters (e.g., vg and capsid titer, filling grade, purity, and aggregation), this process also contains the analysis with a set of suitable orthogonal methods ensuring the utmost level of accuracy and reliability of the data.

In terms of capsid titer determination, our ELISA still represents one of the most frequently used industry standards due to its robustness and accuracy of measurement. However, the evolution of new biophysical methods such as Size Exclusion Chromatography Multi-angle Light Scattering (SEC-MALS) and Dynamic Light Scattering (DLS), or alternative immunoassays, e.g. Biolayer-Interferometry (BLI), and Capillary Electrophoresis (CE) enables the obligatory, orthogonal measurement of AAV capsid titers. Here we present data of blind measurements of our eGFP-filled AAV standards with the methods mentioned above for comparison of independently determined total capsid titers and to underline the reliability of our established characterization process.

The capsid ELISA, SEC-MALS, and BLI showed low assay variation resulting in CVs of around 5%, while CE and DLS showed higher CVs of 6-14% and 10-35%, respectively. Compared to the capsid titer determined by ELISA, each method showed a serotype-dependent average deviation. In summary the average deviation across serotypes compared to ELISA was 13% for SEC-MALS, 46% for DLS, 16% for BLI, and 19% for CE.

The use of reliable standards within diverse orthogonal methods ensures not only the most accurate result but also the safety of the final product in compliance with current regulatory requirements. Therefore, we present the reliability of our characterization process for AAV standards, thus demonstrating their reliability and suitability for biophysical and immunological applications, respectively.

P0196

Fluorescently-labeled AAV Antibodies for the Detection of Intact AAV Capsids

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Recombinant adeno-associated virus (AAV) vectors have become leading tools for viral gene therapy. However, some biological mechanisms are still not completely understood. To better understand the AAV biology, e.g. fluorescence microscopy is a crucial tool to enable the clarification of fundamental questions. For example, the localization of AAVs within the cells, or specific interactions and co-localization, e.g., with host cell proteins can easily be visualized and analyzed. Thus, fluorescently-labelled AAV antibodies that detect only intact AAV capsids facilitate the investigation of e.g. viral life cycle, virus–cell interaction, endocytic pathways, disassembly, nuclear processing and virus assembly.

We've conjugated our widely used and well described anti-AAV mouse monoclonal antibodies A20R, ADK8 and ADK9-1R with the fluorescent dyes AF-488 and AF-647 to provide the fluorescent tools detecting the intact capsids of the AAV serotypes AAV2, AAV3, AAV8, AAV9 and AAVrh10.

Here, we show characterization data of our new fluorescently-labelled AAV particle antibodies, including cross-reactivity, stability and their application in immunocytochemistry. With these antibody conjugates we provide further important analytical AAV tools to be used in development, manufacturing and analytical QC processes of AAV vector development.

P0197

Two for one: A single QC assay to quantify two plasmid impurities (cap/kanR) across a number of serotypes reduces the time and costs for rAAV batch release

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Plasmid derived DNA impurities present in recombinant adeno-associated virus (rAAV) products arise during the manufacturing process and are undesirable byproducts. More importantly, they pose a risk to patient safety and could trigger immune responses impacting product efficacy and long-term expression. Thus, regulatory authorities require thorough analysis of these impurities. As there are different sources of DNA impurities, multiplex ddPCR assays are an excellent way to monitor more than one impurity in a single assay reducing the cost and time of rAAV batch release.

Two critical plasmid derived impurities, usually quantified during rAAV release testing, are packaged AAV capsid gene (cap) and bacterial plasmid backbone derived kanamycin resistance gene (nptII, kanR) sequences. Ascend has developed a duplex ddPCR assay with specific primer/probe sets to simultaneously quantify cap and kanR sequences in one reaction. While the kanamycin resistance gene mainly stays the same between different production plasmids (nptI or nptII), the AAV serotype may change for different productions and projects. Our universal cap primer and probe design detects common serotypes including AAV1, 2, 3B5, 6, 8, 9, hu37, rh10, rh74, and any engineered capsids carrying the respective target sequences such as LK03. Since the primer probe sets are designed to recognize the same position of mispackaged cap fragments, our approach guarantees a good comparison of impurity data between different serotypes. Quantifying the same impurity gene position is the most appropriate comparison between different serotypes and/or production platforms since not all mispacked DNA species are full length. Data generated with our proprietary LR-NGS platform as well as data from assays for the analysis of length distribution of packaged DNA supports this approach and our primer design strategy. Qualification of this universal duplex assay has been performed and confirms a robust and linear working range for both impurities over four log levels with high precision, independent of the serotype.

Assessing cap and kanR impurities in one ddPCR assay enables a faster turnover of results and reduces the costs and sample volume needed for rAAV batch release. Additionally, very little

assay development is needed for new serotypes to be analyzed and the method can go directly into product specific validation to be ready for release testing under GMP.

P0198

A new EM method for determining AAV empty-Full capsid ratio based on drying of AAVs

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Full to empty ratio of AAV delivery vehicles define the potency of gene therapies and different analytical methods have been established to qualify products, manufacturing and processing workflows. The applied method portfolio can be distinguished into methods which determine macroscopic physical properties of the entire sample followed by a mathematical deconvolution based upon various theoretical models, and into model free approaches which are often based upon visual inspection of individual particles followed by the statistical classification in a bottom up approach.

Electron microscopy is very established to visualize, count and determine the full-empty ratios. Cryo-electron microscopy (cryo-EM) has become the gold standard, as it enables the visualization of single capsids in a vitrified-hydrated state without the need for staining. This approach is elaborative and the throughput restricted, which limits its application in daily practice. We experimented and validated an alternative preparative method and like to share our insights gathered with a novel and robust TEM methodology. Instead of freezing, drying of the AAV sample allows us to see in the EM contrast based on the particle density. This methodology was tested for different serotypes and so far shows reproducibility and precision. With the current software and processing solutions, detection of intermediately full particles is not possible, but this avenue remains to be explored further. Even without this capability, this method is much faster while relying on more simple equipment, making it suitable for use on a bench top EM, and allowing for efficient and consistent E/F ratio determination.

P0199

Adeno-associated viral (AAV) vector biodistribution: a bioanalytical approach

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Gene therapy has demonstrated its potential to deliver life-changing treatments to patients, with recombinant AAV vectors emerging as therapeutic products. As with all pharmaceuticals, assessing the biodistribution and persistence of viral vectors is essential before any clinical testing. Many molecular approaches only characterize tissue or biofluid extraction during method validation, often accepting substantial variability in recoveries, typically between 20% and 120%. Additionally, final results are often reported without correcting for the method's recovery rate,

relying solely on PCR results. This study presents the development of a PCR-based method for detecting and quantifying AAV8 viral DNA in biofluids and tissue samples. The method involves extracting viral DNA from biodistribution samples and quantifying it using a selective and sensitive molecular technique, such as qPCR and dPCR. The development process focuses on three main steps: PCR method development, viral DNA extraction, and full workflow optimization.In the first step, we designed and tested over five sets of primers and probes targeting a unique plasmid region, using four commercially available master mixes to identify the optimal combination for amplification efficiency, lowest limit of quantification (LLOQ), limit of detection (LOD), and minimal background noise. In the second step, we spiked AAV viral particles into biofluids and tissue homogenates to simulate real samples. For each biofluid and tissue type, we evaluated at least three extraction kits for their viral DNA recovery efficiency, combined with qPCR and dPCR detection. Despite claims of high extraction efficiency, significant variability was observed. We tested various surfactants and additives to enhance recovery, ultimately discovering a unique recipe that achieved over 80% recovery across all matrices.Among the tested extraction kits and additives, we selected the best one for each biofluid and tissue type, considering viral DNA extraction efficiency, processed sample volume, and final elution volume. In the final step, we integrated the entire workflow into an accurate and precise bioanalytical method. The viral particle was used to as reference material to make standards and QC samples in the respective matrices and extracted alongside samples. This approach resulted in a robust, precise, and accurate method for analyzing biodistribution samples, supporting gene therapy drug development in pre-clinical studies. This novel bioanalytical approach and workflow promise to deliver better results for biodistribution studies by overcoming the challenge of low recoveries from extraction kits and ensuring accurate and precise results for each sample, taking into consideration the entire variability of the method.

P0200

Potential biases when quantifying residual host cell DNA: An 18S rRNA problem

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Minimizing the presence of key residual contaminants is pertinent to the development of Adeno-Associated Virus (AAV) production and purification processes. Host Cell DNA (hcDNA), fragmented DNA from cells used to manufacture AAV product, is a primary residual contaminant. Residual hcDNA is typically quantified by PCR targeting various genes, with the human 18S rRNA gene being the predominant industry target. In this study, we evaluated the abundance of the 18S rRNA gene in both purified Human Embryonic Kidney cell genomic DNA (gDNA) and AAV products manufactured using the same cell line. Our initial results demonstrate that, for two independent AAV products with an identical gene of interest (GOI), the 18S rRNA gene is encapsidated at a level higher than is to be expected when taking into account gene copy number and abundance measured in gDNA. Following this finding, we compared the 18S rRNA results to additional gene targets, including RPLP0, IPO-8, and Adenovirus Type 5 (Ad5) E1A. RPLP0 and E1A genes were found to be encapsidated at an expected level, while the IPO-8 gene was packaged at a level lower than expected. In conclusion, residual hcDNA quantification of AAV product utilizing the 18S rRNA gene may overestimate the amount of hcDNA present. However, as demonstrated by IPO-8, any gene target may inaccurately predict the amount of hcDNA present. Therefore, the results indicate a need to evaluate multiple gene targets and to select a target that is suitable for the specific AAV product and/or production and purification processes. CA Guion, KG McGarry, S Bhattacharyya, SA Hinger, WM Fountain

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P0201

Derivation of satellite cell-specific AAV gene therapy

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The gene Pax7 is a master regulatory transcription factor of adult satellite cells. However, regulatory sequences that drive its expression in vivo have never been described. We have used epigenetic data generated in the Relaix team, as well as publicly available datasets to identify 8 different putative enhancers of this gene. Luciferase assays in murine and human cells revealed activity for only two of these sequences.

In parallel, we have also generated AAVs containing distinct enhancer sequences from this and other satellite cell-specific loci, with the goal of producing vectors for cell type-specific gene delivery. We have found important differences in the ability of specific enhancer-promoter combinations to drive expression in satellite cells in vivo. Our approach holds real promise for identifying satellite cell-specific enhancers, and also using such enhancers for future targeted gene therapies.

Key words: AAV, Satellite cell, Pax7

P0202

Improving rAAV production: key adenoviral elements and their impact on vector yield and quality

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Recombinant Adeno-associated viruses (AAVs) are one of the most used viral vectors for gene therapy, with several products already on the market (e.g., Hemgenix, Roctavian, Elevidys). rAAVs present a favorable safety profile, ability to mediate long-term transgene expression, and broad tissue tropism. The successful approval of gene therapies using rAAVs has increased their demand, placing pressure on existing production strategies to deliver higher titers and better-quality rAAVs. The development of stable AAV producer cell lines without replication-competent virus has emerged as a promising alternative, offering enhanced biosafety, consistent, and cost-effective vector production. However, current strategies have not yet achieved titers comparable to those obtained with transient transfection-based production. We believe that a deeper

understanding of the biological processes involved in the intracellular production of rAAVs can lead to the development of more efficient production platforms.

This study revealed crucial adenoviral factors influencing rAAV production by transient transfection. Starting with an Helper plasmid with full AdV genes (E2A, E4, and VA RNAs) several constructs with varied gene combinations were generated. Their impact on serotypes 2, 5, 8, and 9 productivity was evaluated through transient transfection, in suspension conditions and compared relative to a helper control plasmid. The resulting rAAV vectors were collected and characterized by ELISA (total particles, TP), qPCR (viral genomes, VG), and transducing units (TU). Results revealed that E2A-DBP, E4orf6, and VA RNA alone were insufficient for successful rAAV production, resulting in significant decreases for rAAV2, rAAV8 and rAAV9. Notably, rAAV5 was less affected. Restoration of production levels required specific promoter and region elements, such as the L4P promoter, L4-22/33k, and E4orf3 regions. A smaller plasmid, without adenoviral structural proteins was generated, allowing a similar or better rAAV production across all tested serotypes.

The knowledge generated from this study will ultimately facilitate the development of improved strategies for the large-scale production of high-titer and high-quality rAAV vectors, thereby advancing the gene therapy field.

P0203

Increasing AAV yield: High-Throughput screening of next-gen small molecule enhancers and process automation in cloud-connected bioreactors

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Cell and gene therapies are key innovative modalities for the treatment of cancer, neurodegenerative, and rare diseases. However, the high manufacturing cost of viral vectors is limiting global supply of clinical grade and commercial products, thus reducing access to life-saving therapies for those who need them. To mitigate these challenges, many therapy developers seek process optimization and new technologies to improve product yield.

Virica Biotech and Culture Biosciences collaborated to validate the use of small molecule viral sensitizers (VSEs) for enhanced production of AAV vectors in Culture Biosciences bioreactors. Virica leverages core expertise in high-throughput methodology, advanced automation and robotics tools to streamline VSE molecule discovery. A bioactive compound collection was screened and analyzed for statistically significant enhancement in AAV titer. Several new candidate molecules across a range of chemical structures and diversity were identified for enhanced production of AAV in suspension HEK293 platforms.

Here we describe the validation of these next-generation VSEs for viral vector yield optimization and process transfer and automation in cloud-connected bioreactors. Enhancements in AAV2 yield (vg/mL) ranging from 2-5 fold were validated at 250mL bioreactor scale. Combining the unique technologies of Culture Biosciences' proprietary fully characterized scalable bioreactors and Virica's innovative, effective VSEs, we enabled a high-throughput additive screen and rapid process optimization to set the foundation for a high-yield manufacturing process.

P0204

Determination of genome titer and integrity of adeno-associated virus (AAV) reference standards using the QIAcuity® Digital PCR System

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Recombinant adeno-associated virus (rAAV) vectors are widely used as vehicles for gene therapy in both clinical and research applications. To help ensure product safety, reference standard materials (RSMs) are key. The use of viral vector RSMs is particularly important to normalize laboratory internal controls, for the calibration of medical products and procedures and as best practices for manufacturing and testing of ATMPs. Additionally, AAV RSMs are often used for assay validation and optimization.

All in all, the use of well-characterized AAV RSMs and orthogonal methods is indispensable to meet current authority guidelines. Since the production of such is not achievable for a large number of companies, it is important to resort to commercially available AAV RSMs for the most commonly used AAV serotypes. These standards underlie a comprehensive characterization process including determination of vector genome and capsid titer, filling grade, purity and aggregation. One of the critical quality attributes needed for AAV reference materials is the viral vector genome titer which is usually determined using qPCR.

Here we demonstrate a digital PCR-based streamlined workflow for quantification of genome titers of commercially available, high-quality rAAV reference standards that allows to complete the characterization with increased precision, accuracy and robustness. Additionally, the integrity of the viral vector genome can be assessed ensuring quality and safety of the final product.

P0205

An analytical platform for process and product characterisation of rAAV gene therapies

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One of the main challenges in gene therapy manufacturing is the development of analytical assays and technologies for accurate therapeutic product quality characterisation. With the increasing number of clinical trials for recombinant adeno-associated virus (AAV) gene therapies, a better characterisation of these therapeutic products is needed, not only for final product release but also during the production process to enable manufacturing innovation to meet future patient demand.

Cell and Gene Therapy Catapult (CGTC) has developed an analytical platform for process and product characterisation of rAAV gene therapies by identifying quality attributes with posterior development and assessment of relevant analytical assays and technologies. These analytical

methods have also been developed considering other industry challenges such as accuracy, reproducibility, turnaround times and throughput, some of these tackled through automation. In addition, CGTC has the capability of customising this analytical platform to any rAAV therapy.

Building on several years of investment in the development of improved bioprocessing and automation, CGTC has also created a Process Analytical Technologies lab that is dedicated to enable the rapid generation of detailed and automated process understanding. From this detailed understanding it becomes possible to derive verifiable critical process parameters more quickly, around which create rapid feedback loops using in-line and at-line technologies to control product quality.

P0206

Optimising AAV vector quality and characterisation using synthetic, enzymatically produced linear DNA

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1: 4basebio

As new forms of template DNA become available for Adeno-Associated Virus (AAV) manufacturing, robust analytical characterisation is essential to ensure the quality of AAV vectors produced. 4basebio has developed a proprietary, scalable enzymatic synthesis process for the production of linear closed DNA constructs via its Trueprime™ amplification technology. The hpDNA™ produced is devoid of any bacterial backbone and circumvents cumbersome fermentation processes required for plasmid DNA, while avoiding contamination from endotoxins or host proteins. It also allows unprecedented conservation of notoriously unstable Inverted Terminal Repeat (ITR) sequences, which are prone to recombination using conventional plasmid fermentation. The process is size and sequence independent, facilitating large scale production of GMP grade linear DNA with high yield and purity with significantly shorter turnaround times. Here, we compared the production of AAV vectors using hpDNA™ encoding the typical Adenovirus helper functions, *rep* and *cap* genes, and an expression cassette consisting of AAV2 ITRs and a reporter gene driven by a ubiquitous promoter with conventional plasmid triple-transfection as a control. We focused on validating methods that take in account the fundamental difference of this new template with plasmid DNA to ensure the accuracy of our data, including physical titers and examination of packaged DNA. While achieving equivalency in viral genome titres, full:empty ratios and infectivity between the two production methods, we also demonstrated AAV vectors made from hpDNA™ were generally of better quality, which could greatly accelerate therapeutic development of gene therapy programmes.

P0207

Rational design of viral vector formulations to overcome instability and aggregation during manufacturing

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Viral gene therapy has become a clinical reality for a broad range of both acute and chronic diseases. Approved for various conditions including cancer, metabolic, neurological, and ophthalmological diseases, viral-based drugs, particularly adeno-associated viruses (AAV), stand out in the gene therapy market. These non-enveloped viruses are favored for their genetic simplicity, cell specificity, and long-term gene expression.

As viral therapeutics gain momentum, challenges in manufacturing have emerged, especially in ensuring vector stability and maximizing recovery during the shift from bench-scale to clinical production.

Formulation development for novel biologics like AAV faces unique challenges due to limited preexisting knowledge compared to more established molecules. Each manufacturing step requires tailored formulation optimization to ensure proper functionality of the processing unit. However, some physicochemical conditions in these units results detrimental for the processed biological material, negatively impacting its stability and function.

Use of more mature formulations have the potential to mitigate those detrimental conditions and improve vector stability throughout the downstream processing, but the current AAV formulation toolbox lags other biologics. With only 16 reported final formulation excipients versus 120 for other biologics, expansion of the current formulation toolbox is imperative to enable optimization and enhanced stability of AAV vectors.

Using a comprehensive suite of biophysical and molecular biology analytical techniques, we give insights of the impact of critical physicochemical parameters including temperature, pH, and ionic strength on the stability and potency of AAV2 vectors.

Our findings indicate that certain physicochemical factors have a more pronounced impact on particle aggregation and functionality than others. From these factors, acidic pH fluctuations appeared to have a detrimental role for AAV2, by reducing the melting transition temperature of the capsids and affecting its potency. While under alkaline conditions the melting temperature was also diminished, functionality of the particles was still present.

We also documented the impact of temperature fluctuations on particle aggregation. AAV2 hydrodynamic radius increased directly proportional as temperature. In contrast, increase in particle size was not reported when tracked at a constant temperature for 96h period. This indicates AAV2 aggregation can be described as a function of temperature fluctuations rather than due to exposure at one constant temperature.

Furthermore, we validated the prior observations regarding the impact of ionic strength on AAV aggregation, demonstrating the formation of AAV2 aggregates at low ionic strength. Importantly, these aggregated particles reverted to a monomeric state upon an increase in the ionic strength of the buffer.

Finally, our study unveils the discovery of additives that enhance the thermostability of AAV2 particles and bolster their resilience to exposure to acidic pH. Of note, our results indicate that the augmentation of capsid thermal melt temperature, facilitated by these additives, does not seem to directly correspond to improvements in any of the tested physicochemical factors. These findings provide valuable insights into the intricate interplay between the stability of viral vectors and their physicochemical surroundings.

P0208

Ensuring Genome Integrity Of Recombinant AAV Vectors Using Digital PCR

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Development of safe and effective cell and gene therapies is key to potentially treating a wide spectrum of diseases. Viral vectors have become powerful delivery vehicles for gene therapies. Adeno-associated virus (AAV) vectors have turned into primary modalities for efficient gene therapy applications due to their lack of pathogenicity and persistent transgene expression. Besides the requirement to quantify viral vector genome titers accurately and reproducibly, it is essential to determine the intactness of the viral vector genomes for a safe, stable and effective therapy. Errors made during the replication and packaging process of recombinant AAVs can lead to heterogenous viral vector populations with direct impact on their efficacy and safety. Current purification workflows can efficiently separate empty from full capsids. However, removal of capsids carrying partial or truncated genomes, as well as capsids packaged with host cell or plasmid DNA, are difficult to separate and can be present in the viral vector product after purification. Traditionally, genome integrity has been determined via agarose gel electrophoresis and Southern blot. Next-generation sequencing approaches have also been used to characterize the capsid content. Nevertheless, high resolution and the accuracy needed for integrity determination, as well as repeatability and high-throughput capabilities, remain unmet needs. Of late, multiplex digital PCR (dPCR) has been adopted for genome integrity analyses. Digital PCR enables absolute quantification with unprecedented precision and a higher tolerance towards inhibitors without the need for standards. Here, we propose a rapid dPCR approach for characterizing genome integrity of in-process and purified AAV samples using the same primers and probes that have been optimized for vector genome titration. The underlying Poisson distribution of dPCR enables the assessment of genome integrity over a broad dynamic range by differentiating between physically linked and unlinked targets. As the calculation estimates the concentration for all present groups of template molecules within a sample individually, it could also be used for other applications, such as determination of integrity and stability of DNA and plasmids after certain processing procedures (e.g., restriction enzyme efficiency) or storage. We show that up to five targets can be analyzed simultaneously, increasing precision and reproducibility of the analyses.

P0209

Enhancing the upstream performance of adeno-associated virus (AAV) vector manufacturing via multivariate data analysis

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1: Viralgen

Viralgen as a leading AAV Contract Development and Manufacturing Organization (CDMO) has built a large proprietary data set based on the production of more than 1000 batches produced

across multiple scales and constructs using the Pro10 production platform. The goal of our work is to improve the AAV upstream process by identifying with our proprietary data set which parameters exert the most significant impact on titer.

Process variability and high number of process variables create hurdles for the optimization of the manufacturing process. The first results mainly from the nature of biological processes, accuracy of measurements, and manually operated processes; whereas the latter is implied by the hundreds of simultaneous measurements collected for each sample. Traditional univariate or bivariate techniques fail to simultaneously catch the relationships between many variables.

To address the optimization goal with the associated challenges mentioned, dimensionality reduction approaches were applied, and a multivariate data model was developed using data retrieved from 89 historical batches. The model estimates the relationship between the important process parameters (e.g., pH, metabolite concentration…) and titer at the end of the upstream process as response variables.

We believe our findings reveal a significantly negative impact of viable cell density (VCD) on the vector titer. Dissolved oxygen levels on the day of the bioreactor inoculation, and pH and glucose levels before transfection have also been shown to play crucial roles in batch performance. An experiment explored different VCD levels beyond the current process's operational range, confirming the high impact of VCD and aiding to identify its optimal level regarding titer. In addition, it may show that lower viable cell density results in lower pH and glucose remnants right before transfection.

To integrate the multivariate analysis in daily operations in Viralgen and to bridge the gap between Data Scientists and process experts, we developed an application which identifies contributing factors to the titer of a historical batch in comparison to others in an automated way. This tool will be utilized by the Manufacturing Sciences & Technology Manufacturing and R&D scientists to review the performance and identify potential process improvements in a continuous fashion.

Future enhancement of the model includes the addition of transfection process information and the alignment of offline measurements of different batches in time among others. In addition, we plan experiments to explore the findings derived from a low number of observations and understand their root cause.

P0210

CRISPR/Cas9 editing to generate rAAV-integrated stable clones for Integration Site Analysis methods development

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Viral vectors based on adeno-associated viruses (AAVs) have been widely exploited for gene therapy applications, due to the stable expression of the transgene in non-replicative tissues and lack of pathogenicity. AAVs are non-integrative viruses that mainly remain episomal; however, integration events have been reported. In recombinant AAVs (rAAVs) used for gene therapy, most of the wild type AAV genome is replaced with a therapeutic gene cassette, resulting in an even

lower integration rate. Nevertheless, concerns regarding the potential risk of insertional oncogenesis of rAAVs have been recently raised. In preclinical studies, administering rAAV to neonatal mice resulted in the occurrence of Hepato-Cellular Carcinoma (HCC), which was linked to viral insertion into the Rian locus. Although tumor formation has not been observed in adult mice (unless in presence of other HCC-related risk factors, such as fatty diet or hepatectomy) nor in other animal models or in clinical studies, a proper genotoxicity assessment for human application is needed. Several methods to identify integration sites have been developed and successfully used for integrating viruses. In contrast, the assessment of the integration profile for (r)AAVs presents greater challenges, because of the low integration frequency and occurrence of viral genome rearrangements. Moreover, the lack of well characterized positive controls precluded a direct comparison between different techniques. AAV integration is considered random and more likely to occur in genomic double strand DNA (dsDNA) breaks. In this work, we harnessed this observation to generate a positive control with a defined integration site. Therefore, to drive viral integration in the MCF10A cell line, dsDNA breaks were induced in the PTPN12 gene via CRISPR/Cas9 mediated cleavage while, at the same time, cells were transduced with a rAAV expressing GFP. Subsequently, single cell cloning was performed, followed by clone selection based on GFP expression. Cells were expanded for ~2 months to dilute the episomal AAVs and, thus, select only for clones stably expressing the transgene due to integrated virus. In total, 18 stable cell lines were generated and thoroughly characterized. Vector Copy Number quantification by digital droplet PCR using assays against bGHpA and GFP identified homozygous and heterozygous clones. A discrepancy between bGHpA and GFP measurement was observed in four clones, indicating the presence of partially recombined viral genome. This finding was confirmed by long-range PCR (lrPCR) spanning the PTPN12-cleaveage site. Interestingly, lrPCR showed viral integration in the PTPN12 locus only in 55% of the clones (10/18), suggesting the occurrence of Cas9 editing independent integrations. If confirmed, this finding has clinical implications as cautions should be used when assuming targeted dsDNA breaks will avoid random integrations. The clones hereby generated will be used to assess specificity and sensitivity of different methods, e.g. Ligation-mediated-PCR and Target-Enrichment Sequencing, with or without a background of episomal AAV to simulate a real case scenario in which rare integrated sites may be masked by abundant episomal DNA.

P0211

Implementing dbDNA technology in rAAV manufacturing

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Recombinant AAV (rAAV) has emerged as the lead vector of choice in the development of *in vivo* gene therapies. However, manufacturing challenges relating to productivity and quality form a significant hurdle in clinical product development. Production plasmids that host the viral genes necessary for synthesis are key starting reagents but can be prone to variability in terms of quality, production yield and manufacturing reproducibility. We have therefore explored doggybone DNA (dbDNA™, Toughlight Ltd) as a more cost-effective and scalable alternative to conventional plasmid DNA. Doggybone DNA is a linear, double stranded, covalently closed form of DNA that is ideally suited for biomanufacturing thanks to its minimal size, lack of plasmid backbone sequences and enzymatic manufacturing procedure.

In this study, we investigated the performance of dbDNA in producing rAAV batches with high titres and improved packaging efficiencies. Using a Design of Experiment (DoE) approach, we optimised the transgene, packaging, and helper dbDNA ratios, the total DNA concentration, the DNA/transfection ratios and the transfection mix conditions for transient transfection in HEK293 suspension cultures. Following optimisation, we showed enhanced rAAV productivity with a 1.5-fold increase in vg titre and 3-fold increase in % full capsids compared with conventional plasmid DNA-based production methods. Vg titres and % full capsids were measured using ddPCR and ELISA, and were confirmed by SEC-MALS and mass photometry. In the identified optimised conditions, lower quantities of DNA and transfection reagent were required, emphasising also the economic efficiency of using dbDNA. Nanopore sequencing of the purified capsids manufactured using dbDNA also indicated that the amount of contaminants was 3-fold lower compared with conventional plasmids. Our study shows that when optimised, dbDNA is a more economical alternative to plasmid DNA for the manufacturing of rAAV. The decreased presence of contaminants compared with currently available production plasmids suggest that dbDNA may offer advantages from a safety and therefore regulatory perspective. dbDNA has the potential to greatly increase the manufacturing scalability and purity of rAAV, paving the way for more efficient and cost-effective rAAV-based gene therapies.

P0212

Upstream open reading frames for precise titration of transgene expression in AAV-mediated transduction

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Adeno associated virus (AAV) vectors represent the most frequently used viral gene transfer platform for *in vivo* gene therapy. In the context of high transduction efficiencies and dose sensitive transgenes, it becomes critical to precisely titrate transgene expression levels, and at the same time maximally reduce the variability in expression levels between cells. This underlies achieving biologically relevant expression levels in the highest possible number of successfully transduced cells. Here, we describe the application of upstream open reading frames (uORFs) in the vector genome to efficiently titrate transgene levels while at the same time reducing transgene expression variability between individual cells and independently transduced tissues. To show this, we use expression vectors containing GFP under a CMV promoter either with or without a uORF consisting of an out-of-frame start codon with an optimal sequence for initiation of translation (Consensus sequence: G/ACCATGG) that is placed directly downstream of the promoter. Transfection efficiency of HEK293T cells was retained while overall transgene expression was reduced. Furthermore, by modifying the translation initiation sequence of the uORF, it is theoretically possible to precisely titrate transgene expression levels across a range from 10% to more than 90% of the level of expression without uORF. To demonstrate this, we changed the translation initiation sequence of the uORF from the consensus ACCATGG to TCTATGG and cloned it in an AAV-ITR vector with nano luciferase fused to H2b-GFP by the self-cleaving peptide P2A under the cardiac muscle troponin promoter. These vectors were then used to transfect neonatal rat ventricular myocytes (NRVM). Visual inspection confirmed consistent transfection efficiencies between all plasmids but lower GFP intensity. A luciferase assay performed on the cell lysate confirmed the approximate 80% reduction in protein expression by the ACC uORF while the TCT uORF reduced expression by 50%. These results further show the cell type, promoter and transgene independent ability of uORFs to titrate transgene expression. Furthermore, the uORFs also reduced the coefficient of variation from 9.1% in the control uORF

to 5.0% in the ACC uORF, indicating a stabilizing effect of the uORF on protein production, thereby leading to a more predictable outcome. To demonstrate the potential of uORFs to effectively titrate dose-sensitive transgenes in AAV-mediated gene therapies *in vivo*, we used T-box transcription factor 18 (TBX18) as an example of such transgenes. Inter myocardial injection of an AAV6 encoding TBX18 with and without uORF shows the potential of the uORF to mitigate TBX18-related fibrosis in mouse hearts, reducing total fibrotic area from 15% to 1%. Well-titrated TBX18 expression can be used for transcriptional reprogramming, while high TBX18 expression leads to cardiac fibrosis. We believe that this technology holds significant value also for a broad spectrum of transgenes that would benefit from better control over transgene expression.

P0213

Novel Solutions for Quality Control in Gene Therapy Viral Vector Development

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Interest in gene therapy-based disease prevention and treatment has grown rapidly over the last decade. While lentivirus has historically been the viral vector of choice for gene therapy, recombinant adeno-associated viral (rAAV) vectors have seen widespread application in recent years due to the non-integrating ability. Baculoviruses are emerging as a highly adaptable vector with a broad tissue and host tropism. Regardless of vector, extensive quality control (QC) throughout the entire development and manufacturing process is essential. A robust QC process expedites safe and effective commercialization of the final product. While Sanger sequencing can be ideal to verify and validate plasmid sequences pre-packaging, next generation sequencing (NGS) combined with post-viral production methodologies such as CE analysis and transmission electron microscopy offer an effective high-throughput approach for monitoring AAV quality, from initial construct assembly to analysis of the encapsulated product. Both Illumina® short-read and PacBio® long-read sequencing technologies offer distinct advantages including sequencing of the entire viral genome, with detection of potential mutations, truncations, and contaminants.

In our work we describe our novel proprietary vector-agnostic workflows, for use in lentiviral, AAV or baculovirus settings. Starting with high quality synthesis of either vector plasmid or region of interest, full-length plasmid sequence is confirmed. Depending on these results, correction or new synthesis of plasmid options are applied. Following packaging within the viral vector system of choice, QC results using both short- and long-read NGS platforms are supported with a regulatory-compliant Sanger assay. State of the art synthesis reduces potential upstream errors. Sanger ITR sequencing and sequence correction upstream alleviates potential downstream issues in viral packaging. High-quality viral packaging ensures robust viral titers for downstream use. The combined NGS approach post-packaging alleviates current constraints for high throughput AAV sequencing and thereby enhance the overall QC process. Our Good Laboratory Practices (GLP) Sanger sequencing method extends read lengths through the entire ITR regions, allowing for rapid sequence confirmation of the final AAV product. The combination of these approaches enables a comprehensive solution, ideal for sequence confirmation of both transfer plasmid and final packaged product for improved viral vector gene therapy manufacturing in advance of FDA or EMA filings.

P0214

Stabilizing ITR elements in AAV transfer plasmids

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The Inverted Terminal Repeats (ITRs) of AAV are a critical feature for the rescue of the AAV genome from the transfer plasmid containing the transgene and for efficient rAAV genome packaging into capsids. These are complex palindromic sequences prone to secondary structure formation and often tend to acquire deletions during bacterial propagation of transfer plasmids. Such plasmid populations with a fraction containing truncated ITRs seriously undermine plasmid homogeneity and cause shortcomings in AAV yield and genomic purity. Full length ITRs are effective in resulting in full capsids and homogeneous payloads. However, they are severely unstable on plasmids under normal bacterial growth conditions. In this study, it was shown that turning to optimized fermentation conditions such as for example, cultivation temperature, growth medium and the choice of the right bacterial strain, containing modifications in genes responsible for genome organization, it was possible to deny *Escherichia coli* the conditions that would potentially lead to ITR sequence deletion. Complementing these experiments is a strong and robust analytical tool to measure ITR lengths in a plasmid population using NGS. Additionally, ITR length variants were characterized using Capillary Gel Electrophoresis and found suitable as a complementary, rapid in-process control method. We thus demonstrate our ability to restore ITR sequences to their full length (wt length) condition, maintain them during cultivation and effectively track them by a robust analytical method. These findings open new possibilities for AAV manufacturers to increase their product yield and quality.

P0215

An optimized protocol to transduce photoreceptors in human retinal organoids using AAV

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In vitro models, such as retinal organoids, are increasingly gaining traction in the evaluation of novel gene therapy strategies. Although we have demonstrated the feasibility of infecting retinal organoids with AAV vectors, the outcomes vary considerably due to factors like age and infection medium, which can influence both the infection level and organoid development. Hence, it is imperative to consider these diverse parameters to optimize expression without compromising organoid development. By employing quantification through RT-ddPCR and subsequent RNA sequencing analysis, we monitored the expression levels and organoid development post-infection. The outcomes of the infection process are notably affected by the presence of serum in the medium, as it can compete with AAV receptors. Additionally, the age at which the infection occurs plays a crucial role. Infecting overly immature organoids may jeopardize their post-infection development, while mature organoids with segments exhibit a reduced propensity to infection due to the presence of a mechanical barrier. Our results highlight the importance of considering seemingly minor parameters, such as age and infection medium, to achieve a successful infection.

P0216

Entry, trafficking and efficacy of AAV8 in human muscle cells from Duchenne muscular dystrophy

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Adeno-associated virus (AAV) serotype 8 is of particular interest as a vector used in gene therapy for neuromuscular disorders, e.g. Duchenne muscular dystrophy (DMD). However, it is well recognized that AAV-mediated transduction in DMD muscles is not optimal as we demonstrated by the lower transduction efficacy in DMD animal models and muscle cells derived from DMD patients compared to healthy controls. In this context, basic knowledge is missing to understand the mechanism of AAV-mediated transduction in diseased muscles, especially regarding the entry and trafficking of AAV which are limiting steps of AAV transduction for many cell types. Our project aims to characterize cellular uptake, intracellular transport and expression efficacy of AAV8 in muscle cells from DMD patients. In this study, we showed that the AAV genome is equally internalized in DMD and control muscle cells while it is less efficient at expressing its transgene in DMD compared to controls cells. We further showed that the universal AAV receptor (AAVR) is less expressed in DMD cells and likely involved in intracellular trafficking of AAV8 to the perinuclear region and, vector maturation. Encapsidated and uncoated AAV genomes were visualized in DMD and control cells by immunofluorescence, electron microscopy and fluorescence *in situ* hybridization (FISH). Our study provided a picture, unknown until today, of AAV transduction efficiency in human DMD and control muscle cells and highlighted potential defect of AAV trafficking and maturation in the pathological muscles which may limit therapeutic benefit in future clinical application.

P0217

Dissecting rAAV production by HEK-293 proteom analysis and cell-free AAV assembly

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Recombinant AAV production is well studied; nonetheless, remaining knowledge gaps in the involved constituents, their interconnection and their relative importance as well as the underlying molecular mechanisms hamper rational process engineering. In a top-down approach, we analyzed the nuclear HEK-293 proteome during triple plasmid (pITR, pRepCap, pHelper) rAAV production. Comparison with control-transfected and untransfected HEK-293 yielded protein candidates, which illuminate the cellular response and warrant the study of their expression control for production improvements. Also, the addition of small molecules inhibitors hinted mechanisms. In a bottom-up approach, we studied capsid formation from purified VP proteins, produced ITR DNA as well as Rep proteins and demonstrated cell-free DNA encapsidation.

P0218

Upstream Process Optimization to Generate Internal Reference Standard Material for rAAV9 Full Capsids: A Step Towards Standardisation of rAAV F/E Characterisation

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Recombinant adeno-associated virus (rAAV) has emerged as the vector of choice for in vivo gene therapies largely due to its capacity for long-term expression and strong safety profile. However, manufacturing still faces challenges such as inaccurate quality control due to the lack of well-characterized reference standard material (RSM) which can serve as a benchmark. The absence of RSM hampers the comparison and correlation of different analytical technologies, which are crucial for establishing standardised analytical protocols for accurate rAAV characterisation. In this study, we aimed to produce internal RSM for full capsids of rAAV9 by optimizing both adherent and suspension-based transient transfection production processes and assessing both methods for total viral titer and full capsids titer (% full). We compared different combinations of helper (pHelper) and RepCap plasmids (pRepCap). Four different pHelper were used, two containing helper genes from Ad5 (pHelper1,pHelper2), one containing helper genes from Ad2 (pHelper3), and one containing helper genes from Ad5, HIV, and human bocavirus (pHelper4). Two pRepCap were used: one with a heterologous MMTV promoter (pRepCap1) and one with a truncated native p5 promoter (pRepCap2), driving rep and cap genes expression. The plasmid ratio was kept equimolar and a PEI:DNA ratio of 2:1 was used. All productions were performed at a small scale, with 10 cm Petri Dishes (adherent) and 25 mL shake flask (suspension). We found that all combinations with pRepCap2 showed higher viral titer and % full titer than combinations with pRepCap1. pHelper4+pRepCap2 gave the best total viral titer and % full titer. Once the optimal plasmid combination was determined three PEI-based transfection reagents were assessed for better viral titer and % full titer. The transfection reagent procured from PolyPlus (Fecto-VIR AAV) was found to provide the best total vector titer and % full titer. We developed an optimized protocol for both adherent and suspension cell cultures to achieve a higher % full yield of rAAV in crude cell lysate. Optimizing the lysis process will be the focus of follow-up research. The final protocol developed after optimization will be published to assist researchers in achieving a high viral titer and % full titer of rAAV9, enabling a ready-to-use streamlined, and efficient process. This study represents a critical step toward the standardisation of Full and empty (F/E) analytics, facilitating faster and more reliable production of rAAV vectors.

P0219

Systematic study of human adenovirus receptor-usage utilizing a human in vitro respiratory epithelial cell model

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Adenovirus vectors are amongst the most extensively studied vectors and they are widely applied in clinical trials. However, ADSTILADRIN, an interferon expressing replication-deficient recombinant adenovirus type 5 vector (also known as rAd-IFNa/Syn3) is so far the only adenovirus-based gene therapy product amongst the over 30 FDA and EMA approved cellular and gene therapy products. In sharp contrast, there are plenty adenovirus types identified from human (116 types), offering great potential to broaden the selection spectrum of vectors for therapeutic utilization. With the high demand on efficiency and safety for gene therapy products, it is crucial to achieve cell-specific gene delivery and to avoid off-target effects. Therefore, the cellular entry receptor profile is one of the selection criteria for non-Ad5 vector development. However, to gain a systematic view of adenovirus receptor usage, a proper human cell -originated model is still lacking. Our aim is to establish such a model for comparative analysis of the receptors used by human adenoviruses in cellular entry. Based on the human alveolar adenocarcinoma cell line A549, we generated a panel of stable receptor-knockout cell lines using the CRISPR/Cas9 technology. These cell lines carry either a CAR, CD46, DSG2 single-, or double- or triple-knockout of all the three major receptors. We then screened our previously established reporter gene labeled-adenovirus library in this knockout cell model. Several methods were applied, including luciferase assay, GFP-based flow cytometry, anti-hexon antibody staining-based flow cytometry and viral genome quantification via qPCR. First, we can confirm the CD46-dependence of human adenovirus type B16, -B21, -B34, -B35 and -B50 with our human in vitro model with the single-, double- or triple knockout. Types C5 and E4 were proven to be CAR-dependent. As to the other species B adenoviruses (B3, B7, B11 and B14), both DSG-2 and CD46 play a role in cell entry. Moreover, we observed a negative effect of CAR-knockout for species B adenoviruses (B3 and B14) and several species D adenoviruses on cell entry. Another interesting discovery with this in vitro model was that fiber-modified adenovirus vectors with increased affinity to DSG2 (mutation junction opener 4) are absolutely DSG2-dependent enabling de-targeting from CD46 which presents on every nucleated cell. Noteworthy, we also observed that triple knockout cells are still comparably permissive to adenovirus type D17, which is a promising candidate for endothelial cells transduction. This suggests that D17 uses another, yet unidentified receptor. With comparative analysis of previous knowledge and our recent data, we are on the way to build up a clear landscape of the human adenoviruses receptor profile. Our in vitro model with adenovirus major receptor-knockout cell lines will enable the identification of the most applicable adenovirus types for individual therapeutic application and to further understand adenovirus infection biology.

P0220

Considerations for AAV analytical comparability studies for products with low batch numbers

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Manufacturing changes are often implemented during the development of AAV gene therapies. These changes may be significant, for example changing the manufacturing platform to generate a scalable manufacturing process, or much smaller, such as transferring an existing process from one manufacturing site to another.

These manufacturing changes must be accompanied by comparability studies demonstrating that the post-change product has an equivalent safety and efficacy profile to the pre-change product. If analytical comparability can be demonstrated based on a good understanding of product critical

quality attributes (CQAs) and using methods that can provide high assurance of safety and efficacy, then repetition of preclinical toxicity or human dose-finding and efficacy studies may not be needed.

Several guidance and draft guidance documents are available from various agencies to guide the comparability process. Ideally, a large number of pre- and post-change batches should be compared to provide statistical assurance that the change(s) introduced do not affect product CQAs. However, since many AAV gene therapies are often produced for rare diseases with relatively low numbers of patients and since batch manufacturing costs are high, a limited number of batches is normally available.

Here we present examples of comparability plans which are compatible with current guidance, and which are tailored to AAV gene therapies. This includes considerations for CQA risk assessments when changes are made to different parts of the process, e.g. upstream, downstream or formulation, as well as strategies for generating data that provides sufficient statistical assurance of comparability using only a small number of pre- and post-change batches.

P0221

Innovations in Size Exclusion HPLC for Efficient Analyses and Insights on AAV Manufacturing

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Gene therapy has advanced over the past decade into a promising therapeutic class for treating many intractable diseases. Recombinant adeno-associated virus (AAV) vectors are currently the leading vector platform. AAV drug products are inherently heterogeneous, containing full, partially filled, and empty capsids as well as aggregates, fragments and other process related impurities. Among other critical quality attributes (CQAs), the levels of AAV fragments and aggregates need to be monitored.

Size exclusion chromatography (SEC) is becoming widely adopted as a platform analytical method for AAV, where it is possible to combine the separation with multiple wave-length UV -, fluorescence (FL) -, multiangle light scattering (MALS) - and refractive index (RI) detectors. The current practice of analytical SEC for AAV analysis is based on the use of ≥ 5 µm diameter 300 to 1000 Å pore size packing materials and large volume columns (i.e. 7.8 x 300 mm) made of stainless-steel hardware. This means that analyses can be time consuming, use significant amounts of samples, and may not have adequate resolution to facilitate a detailed understanding of different size variants and impurities. It also means that the methods can be plagued with non-specific interactions. This affects the quality of the separation and the analyst's ability to characterize and monitor important product-related impurities. The column hardware related interactions mostly occur with columns made of stainless steel. To this end, recently developed hydrophilic hybrid organic–inorganic surface (h-HST) hardware is proving to be useful and effective in improving aggregate recovery for various samples.

In this study, we have explored AAV SEC separations and ways in which we can increase throughput and decrease a dependence on lengthy mobile phase development. As such, a systematic comparison has been performed between various column packed with different 2.5 µm diol-bonded BEH ca. 500 Å particle batches into two different types of column hardware (one based on stainless-steel and the other manufactured to have hydrophilically modified hybrid

surfaces). Shear forces and friction effects on critical AAV measurements have also been systematically evaluated. Lastly, a new simple experimental design has been employed to implement a method robustness. This approach requires only 8 quick experiments to confirm the absence (or presence) of secondary interactions. In sum, a collection of new analytical characterization tests have been optimized to give ample opportunity for Chemistry Manufacturing Control groups to implement process improvements. These multi-attribute size exclusion methods will help give quick insights on manufacturing approaches and thereby make it possible to improve yields, potency, and safety profiles of new and soon to be approved AAV drug candidates.

P0222

Advancing Serological AAV Assays with PROGEN's Human Chimeric Antibody Standards

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Recombinant adeno-associated virus (AAV) vectors are leading tools for viral gene therapy. However, many humans in the general population have developed antibodies against AAV as a result of naturally acquired infections, which might affect efficiency and safety of the gene transfer using AAV vectors. Therefore, testing for pre-existing AAV antibodies in patient sera is an indispensable step for the selection of patients for AAV gene therapy clinical trials. The use of suitable positive controls for the development of reliable and reproducible serological AAV assays is crucial. Therefore, PROGEN developed recombinant human chimeric AAV antibodies based on our exclusive portfolio of neutralizing anti-AAV mouse monoclonal antibodies (see antibody clones in Tab. 1). The antibodies retain the identical variable region compared to the mouse monoclonal AAV antibodies while containing a human Fc region. This allows the detection with an anti-human secondary antibody and ensures comparability with human serum antibodies. Here, we show characterization data of our new human chimeric antibodies, including binding affinity, cross-reactivity and their application in serological assays.

P0223

Establishment of a transient rAAV production platform by a holistic approach - Matching HEK293 cell line, plasmids, and production process

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Recombinant adeno-associated viruses (rAAV) are currently the predominant viral vector for *in vivo* gene therapy in clinical studies. For manufacturing, transient plasmid transfection of HEK293

cells is commonly used. Initially, it is focused on increasing the yield by (1) the selection of a highly productive host cell line, (2) designing a potent plasmid set, fine-tuned for component ratios and (3) the bioprocess and medium/feed used.

We have developed a platform workflow approach that allows automated high throughput screening of 400 single cell derived HEK293 cell lines for rAAV productivity (vg/L and TU/L). Resulting top single cell derived cell lines as well as pools of the original suspension adapted HEK293 were tested with various plasmid sets, rAAV serotypes, transfection reagents, feeds, and media to determine optimal combination and approaches. For screening experiments, Ambr® 15 and the Ambr250 HT were used as bioreactor systems with controlled environment, making it ideal for identifying the best cell clones and setup for later scale up to larger volumes. While yield is highly dependent on the medium, feeds and plasmid ratios; we show that even the impact of the plasmid system varies greatly depending on cell line or even culture conditions. Overall, vg/L depends mostly on the clone, transfection reaction and the plasmid set used. The number of full particles depends on the plasmid set and transfection reagent, as well as feed strategy. Accordingly, optimization of the setup and selection of the best combination of parameters is a highly complex task and requires a high throughput approach ideally applying integrated design of experiments and multivariate data analysis which are part of Sartorius´ data solution platform (e.g. MODDE® and SIMCA®).

P0224

An investigation of the potential of SiNPs particles to enhance Adeno-Associated virus gene transfer

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Adeno-associated virus (AAV) is currently at the forefront of the rapidly evolving field of gene therapy. Nevertheless, there are still challenges with this vector that must be addressed to fully maximize its capabilities and ensure its widespread application. A significant obstacle lies in the elevated dosage of vector needed for gene transfer, leading to increased levels of toxicity observed both *in vitro* and *in vivo*. Hence, efforts to guarantee equal or higher transduction efficiency of AAV while reducing toxicity is crucial.

One approach is to combine viral vectors with enhancer molecules to create hybrid delivery systems that improve vector delivery with equal efficacy at lower vector dose. We investigated the potential of a novel silica nanoparticle (SiNP), Nuvec®, developed by N4Pharma Ltd, to enhance AAV transduction. To do this, we used PEI coated and PEI-free/Nuvec® SiNPs with spike surface topography to improve gene delivery at reduced MOI on target liver cells.

HepG2 cells were treated with AAV2/CMV/GFP/8 at MOIs of 10^5 and 10^4 with and without complexation with Nuvec®. Even though, we observed an increase in transduction with AAV complexed with Nuvec®, the infectivity in these cells *in vitro* was low, as expected. To investigate gene transfer to cells more representative of the liver, 3D hepatocyte-like cells (HLCs) differentiated from induced pluripotent stem cells (iPSCs) were used. We firstly profiled HLCs transcriptomically and found these cells closely align to primary hepatocytes in contrast to HepG2 cells. Next, HLC were treated with different concentrations of Nuvec® and cell viability, survival and AAV transduction was assessed. Optimal viability was observed at 2µg/ml of PEI/Nuvec® and 20µg/ml of PEI-free/Nuvec®. In terms of AAV transduction enhancement, all concentrations of

Nuvec® in presence or absence of PEI showed an increase compared to AAV2/CMV/GFP/8 used alone. Nevertheless, PEI-free/Nuvec® was more efficient than PEI/Nuvec® for gene transfer. With 20µg/ml with PEI-free/Nuvec® at the lower MOI of 10^4, there was a 2.68-fold increase, whilst with PEI/Nuvec® a 1.14-fold increase was observed compared to AAV2/CMV/GFP/8 alone.

Also, the potential of Nuvec® to enhance AAV stability at temperatures other than at -80°C was explored. AAV2/CMV/GFP/8 was complexed with Nuvec® and left at room temperature (RT) or at 4°C for 30 days. AAV transduction levels were lost over time at both RT and at 4°C, however, when complexed with PEI/Nuvec® vector stability improved at both temperatures with the most significant being when complexed with PEI/Nuvec® at 2µg/ml and 20µg/ml at 4°C and RT, respectively.

In conclusion, AAV gene transfer to HLCs can be improved with inert silica spiked nanoparticles to reduce vector dose whist retaining high level gene transfer. Nanoparticles also appear to reduce or even prevent loss of AAV infectivity and maybe considered cost effective and useful for vector transport between manufacture and application.

P0225

Optimization of AAV genome size by stuffer sequences to balance packaging efficiency, genome integrity and bioactivity

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Adeno-associated virus (AAV) vectors currently represent the most attractive platform for therapeutic gene delivery. Ensuring efficient AAV production and vector integrity, defined by efficient packaging of full-size genomes, a high full/empty ratio, and optimal bioactivity, therefore is of utmost importance to facilitate clinical development.

The genome of recombinant AAVs, whose size is limited to approximately 4.7 kbp, minimally exists of a promoter, a transgene sequence and a poly(A) signal, flanked by inverted terminal repeats (ITR). However, it is well known that not only capsids that carry the expected full-size genome of interest, but also empty capsids, as well as capsids containing partial/truncated or oversized genomes, are produced. Steadily rising clinical development efforts, authority requests and analytical advancements (e.g., sequencing technologies and mass photometry) have contributed to a continuously increasing understanding of vector features that impact integrity. However, systematic studies on non-coding “stuffer” elements that can be used to optimize vector genome size, are sparse.

To investigate the impact of AAV genome size on packaging efficiency and genome integrity, we designed single-stranded CMV-eGFP-p(A) expression cassettes, increasing in size from 2 kbp to 5 kbp due to the insertion of consecutively elongated stuffer sequences, placed either upstream (5’) or downstream (3’) of the CMV-eGFP-p(A) sequence. Additionally, we compared two different stuffer sequences. All 5’-Stuffer and 3’-Stuffer designs were packaged into AAV6.2, using HEK-293 cells, and purified by iodixanol-density-gradients and ultrafiltration. For quality control and packaging analysis, total AAV yields were quantified using ITR-, GFP- and poly(A)-specific dPCR. Vector integrity was assessed by DNA gel electrophoresis, sequencing, and mass photometry, to determine the (over)full/partial/empty ratio. Finally, bioactivity was analyzed by transduction of HEK-293 cells.

Our data show a systematic decrease of AAV yields, bioactivity and fraction of overfilled capsids with increasing AAV genome size, for both, the 5' and the 3' stuffer designs. Importantly, the stuffer sequence itself had great impact on AAV yields and bioactivity, as high yields and bioactivity were only preserved with one of the two tested stuffer sequences. Furthermore, our study revealed that small-sized AAV genomes (2-2.5 kbp) are prone to overfilling, whereas large AAV genomes (4.5-5 kbp) are more likely to be truncated, regardless of the insertion site of the stuffer sequence. Notably, the appearance of undesired partial or overfilled AAVs was minimal at genome sizes of 3-3.5 kbp.

In summary, our data demonstrate that genome size is an important parameter, impacting AAV yield, bioactivity and genome integrity. While the insertion of non-coding DNA is a straight-forward approach to "right-size" the AAV genome, our data suggest that the insertion position and sequence identity have a major impact on critical quality attributes and therefore require thorough assessment.

P0226

Investigating the mechanisms of AAV-mediated dorsal root ganglia (DRG) toxicity by single nucleus RNA sequencing in C57BL6 mice

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Somatosensory neurons play a critical role in the sensation and the responses to external stimuli. Sensory neurons have shown to be susceptible to toxicities induced by high doses of adeno-associated virus (AAV) and AAV gene products. This pathology is generally characterised by neuronal and axonal degeneration in NHPs, piglets, rats and others. In this report, mice were shown to exhibit molecular and transcriptomic alterations consistent with findings in prior studies. Wild-type C57BL6 mice were intrathecally dosed with AAV-miR-SOD1 vector (PackGene Biotech) at various doses, animals were then sacrificed for lumbar DRGs used in single-nucleus RNA sequencing. At 3 weeks post-injection, serum neurofilament light (NfL) levels surge in response to the AAV vector. A notable reduction of neuronal (>50%) and Schwann nuclei (~8%) were detected in mice with high serum NfL levels. A new cluster labelled activated satellite glial emerges along with an increase in T cells and macrophage cells. Key developmental and repair associated (axonal guidance) genes were upregulated in activated satellite glia. Innate and adaptive immune genes were upregulated in macrophages and T cells. This study provides valuable cell type specific mechanistic insights in response to AAV-induced DRG toxicity in mice. These findings may aid in designing of immunosuppressant-based mitigation approach to alleviate AAV-related DRG toxicity for improved use of gene therapies in treating CNS diseases.

P0227

Toward a gene therapy platform- what's the best format of TFF?

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As monoclonal antibody (mAb) platforms evolved, the roles for each tangential flow filtration (TFF) format settled into place. In upstream applications we take advantage of the low shear environment in hollow fibers to recirculate cells without impacting product quality. Flat sheet cassettes are typically used in downstream applications, as their higher turbulence enables faster processing in smaller footprints.

However, platform processes for viral vector-based gene therapies are still in their infancy, and the roles for TFF technologies have yet to be defined. Interestingly, we have seen many examples of innovators using different technologies in the same application. These new modalities carry with them unique product and process requirements compared to mAb's. For example, lower product stability may push us towards faster and gentler processing. And more challenging adventitious agent clearance may increase the need for closed processing. With these requirements in mind, is there an 'optimal' TFF option?

Here we aim to address this question with a comparison of TFF technology which includes format (hollow fiber and flat sheet) as well as membrane chemistry. The work focuses on adeno-associated virus (AAV) and includes data from several common serotypes and multiple locations along the downstream process. We will share technical data comparing key performance outputs such as flux, purity, and yield. Included in that work we attempt to define flow and pressure limits based on product quality (i.e., shear sensitivity of the viral vector). Finally, our comparison will cover manufacturing considerations including footprint, process economy, scalability, and options for closed processing.

P0228

Development of an AAV8 shedding assay to support gene therapy clinical trials

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The number of gene therapy clinical trials has steadily increased in recent years. A fundamental requirement for these trials is to monitor vector shedding to control the potential environmental risk associated with viral DNA diffusion and to provide investigators with relevant information about vector-shedding clearance post-therapy. Viral shedding is generally assessed by isolating gDNA from the target matrix followed by PCR analysis. Most labs only evaluate extraction efficiency during method validation, often accepting disappointingly low recoveries ranging from 20% to 120%, with even lower recoveries for urine samples.

Using an AAV8 vector as a model, we developed a workflow for identifying the optimal protocol for analyzing six spiked matrices typically considered in viral shedding studies: blood, plasma, saliva, urine, feces, and semen.

In the first part of the project, we tested five sets of primers and probes and three commercially available DNA polymerases using qPCR and dPCR. Our goal was to identify the most robust and sensitive combination based on qPCR efficiency, the lower limit of quantification (LLOQ), and the limit of detection (LOD). Next, we spiked the six target matrices with the AAV8 vector and assessed viral DNA recovery using seven commercially available gDNA extraction kits. Despite claims of high extraction efficiency, significant variability was observed. By testing various surfactants and additives, we developed a unique recipe that increased the extraction efficiency to over 80% across all matrices. The best kit for each matrix was then selected based on this enhanced extraction efficiency and the theoretical extrapolated LOD.

Finally, we demonstrate that for obtaining the most reliable results, both the standard curve and quality control samples should be prepared in the target matrix and extracted alongside the shedding samples. This approach ensures accuracy and precision in viral shedding analysis, supporting gene therapy clinical trials by providing reliable and sensitive monitoring of vector shedding.

P0229

From chemical to mechanical: A comparative analysis of cell lysis strategies for mammalian cells

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Adeno-associated virus (AAV) gene therapy offers a revolutionary approach to treating a wide spectrum of genetic diseases. However, realizing its full potential hinges on the development of robust and scalable manufacturing processes providing high quality and high potency vectors at commercially viable costs. A critical bottleneck within this process is cell lysis, the controlled disruption of production cells to release the encapsulated AAV vectors. Most AAV serotypes mainly reside within or adhere to the production cells and are not significantly released to the supernatant, and the chosen lysis strategy to harvest rAAV significantly impacts both the yield of AAV vectors and the efficiency of downstream purification steps.

In this study, we evaluated and compared various cell lysis strategies specifically tailored for AAV gene therapy manufacturing. We investigated a range of methods, including:

- Chemical Lysis: This method employs mild detergents or salts to selectively permeabilize the cell membrane, allowing the release of intracellular components without compromising the integrity of the AAV vectors. However, selecting the appropriate chemical and optimizing its concentration are crucial to ensure efficient lysis while minimizing unwanted interactions with the AAV particles. We compared in this study various commercial and in-house cell lysis reagents.

- Sonication: Sound waves are used to disrupt the cell membranes in this method. While offering scalability and relative ease of implementation, sonication can generate significant heat, potentially affecting the stability and functionality of the AAV vectors.

- Mechanical Lysis: This approach utilizes high pressure homogenization to physically disrupt the cell membranes, releasing the intracellular contents. While efficient, it can also damage the delicate AAV particles, necessitating careful optimization of the selected pressure.

We assessed each method's impact on several key parameters. Cell lysis efficiency was evaluated through recovery calculations of the AAV vector yield, determined using digital droplet PCR (ddPCR) and enzyme-linked immunosorbent assay (ELISA) As a follow-up to this study, it is planned to investigate the compatibility of the lysate generated by chemical lysis with that generated by our current gold standard (mechanical lysis) for subsequent downstream processing steps, particularly focusing on factors like lysate viscosity, turbidity, pH and conductivity.

By presenting a comparative analysis of these diverse lysis strategies, we aim to highlight their relative strengths and weaknesses in terms of cell lysis efficiency, product quality impact and potential for large-scale manufacturing. Our current manufacturing platform is strong, reliable, and delivers excellent results. We're also actively developing next-generation processes to push efficiency, quality, and cost-effectiveness even further. Paving the way for wider clinical application and improved patient outcomes.

P0230

Development of a High throughput qPCR analytical platform

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The recombinant Adeno-Associated Virus (rAAV) has been successfully used as a viral vector for Gene therapy trials and represents an interesting tool in the development of new therapies for hearing loss disorders.

The Upstream and Downstream process steps need to be developed for each new rAAV therapeutic candidate to support the preclinical and clinical needs. The process development steps generating a substantial number of samples to characterize the process and the product quality, the development of high throughput analytical platform becomes essential to accelerate the research and innovation in gene therapy development.

Different analytical methods are based on DNA extraction followed by qPCR assay as Viral Genome (VG) titration, residual DNA quantitation (Host Cell DNA and plasmid DNA) and used to define some process parameters such as yield, residual DNA clearance. To speed up the lead time and increase the number of samples per run, a semi-automatize platform has been implemented. Three major steps are performed during DNA quantification by qPCR. First, three independent DNA extractions per sample, followed by three serial dilutions per extraction, qPCR plate preparation and analysis. Automate performing DNA extraction in 96MW plate format compared to the manual DNA purification on columns increases the throughput of DNA extraction from 8 to 32 samples carry on in 50 min instead of 2 hours. Then a liquid handling robot adapted for both 96MW and 384MW plates is used for serial dilutions and preparation of the qPCR plates. The 96MW plates preparation with the liquid handling automate conducts to an important operator time saving (50min handling vs 2h for the manual dilution and preparation plate) and allows the analysis of 8 samples per run. The 384MW qPCR preparation manually is tedious and time-consuming and can lead more often to operator errors. By using the automate, the time saving is

more than 1h and allows the analysis of 32 samples with 1 targeted gene up to 8 samples with 4 different targeted genes. Finally, the entire automate process leads to 32 samples analysis in half-day versus 2 days manually.

In conclusion, the High throughput qPCR platform allows to decrease time operator, to increase both the throughput, the number of samples analyzed and the type of analysis performed on one single run in order to accelerate the process development and product characterization.

P0232

Scaling Up AAV Production: Optimizing Plasmid DNA Complexation for Transient Transfection

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While AAV gene therapy has entered the commercial landscape, achieving scalable and robust manufacturing processes for consistent high-quality and high-yield AAV production remains a challenge. Recently, we developed mammalian transient transfection-based processes for AAV production in single-use stirred tank reactors up to the 1000 L production scale. During this work, we identified two critical aspects of the workflow required to ensure consistent, high-titer, and high-quality AAV production: 1) the health of the cells at the time of transfection, and 2) efficient and consistent transfection. In particular, the complexation of the transfection reagent with plasmid DNA and subsequent delivery to the cells is a critical process step. Developing an in-depth understanding of this step is important to optimize large-scale production processes for performance and reproducibility, as well as minimize risks associated with costly production failures. In the present study, we utilized colorimetric-based studies as well as actual large-scale complexation reactions to identify the critical parameters enabling efficient and consistent complexing of plasmid DNA and transfection reagent in bioprocessing bags and stirred tank mixers. For bioprocessing bags, the parameters evaluated included headspace, rocking angle and speed, orientation of the bag on the rocker, mixing time, complexation volume and pump flow rates. For stirred tank mixers, parameters tested included mixing time and top versus bottom addition of components to the reactor as well as power input. The data from these studies indicate the key aspects of large-scale plasmid DNA complexation and delivery. We rank a hierarchy of factors that should be evaluated and controlled during the transfection process, and we propose a framework for developing complexation protocols using different mixing platforms. Taken together, the results of this work provide a set of guidelines for scaling transient transfection to achieve reproducible high titer AAV production at large scale.

P0233

Precision Cell Engineering: Viral and Non-Viral Vector Solutions for Cell and Gene Therapy Innovations

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The rapid advancement of cell and gene therapy has been significantly propelled by the precise engineering of cells using both viral and non-viral vectors. Viral vectors, such as lentiviruses, and adeno-associated viruses (AAV), offer high transduction efficiency and long-term gene expression, making them ideal for a range of therapeutic applications, including gene editing, CAR-T cell therapy, and the treatment of genetic disorders. Non-viral vectors, including lipid nanoparticle coated RNA and ssDNA, provide safer alternatives with reduced immunogenicity and enhanced delivery efficiency for RNA-based therapies or genome editing. By leveraging the synergistic potential of viral and non-viral vectors, we can enhance the precision and effectiveness of cell engineering, paving the way for groundbreaking treatments for a myriad of diseases.

Here were offer a streamlined, and end-to-end workflow from *de novo* DNA synthesis to **High quality and titer viral vector (AAV and Lentivirus) production** with various solutions such as multiple AAV serotypes, high viral titers and volumes to tackle specific challenges, maximizing the success rate of cell transduction and cell line engineering. We also present our **Single-strand DNA synthesis (linear and circular)** capabilities with 100% sequence accuracy, allowing for more complex genetic modifications and increased flexibility in designing CRISPR experiments.

Furthermore, **our *in vitro* transcription (IVT) RNA synthesis** offers linear and circular RNA with customizable modifications like cap analogs, poly(A) tails, and modified nucleotides. These enhancements maximize RNA stability, translation efficiency, and immunogenicity, optimizing downstream applications of mRNA technologies.

In conclusion, Azenta Life Sciences' synthetics services provide a full end-to-end spectrum of reagents that help accelerate cell and gene therapy programs.

P0234

Is my next generation sequencing deep enough?

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In combinatorial genetic engineering experiments, next-generation sequencing (NGS) allows for measuring the concentrations of barcoded or mutated genes within highly diverse libraries. This is particularly crucial during directed evolution screenings of AAVs. When designing and interpreting these experiments, sequencing depths are thus important parameters to take into account. Service providers follow established guidelines to determine NGS depth depending on the type of experiment, such as RNA sequencing or whole genome sequencing. However, guidelines specifically tailored for measuring barcode concentrations have not yet reached an accepted consensus. To address this issue, we combine the analysis of NGS datasets from barcoded libraries with a mathematical model taking into account the PCR amplification in library preparation. We demonstrate on several datasets that noise in the NGS counts increases with the sequencing depth; consequently, beyond certain limits, deeper sequencing does not improve the precision of measuring barcode concentrations. We propose, as rule of thumb, that the optimal sequencing depth should be about ten times the initial amount of barcoded DNA before any amplification step.

P0235

Comprehensive characterizations of adeno-associated virus vectors -Analytical method development and application to AAV drug product

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We have been developed analytical methods for comprehensive characterizations to ensure efficacy and safety of AAV vectors. Multiwavelength detection sedimentation velocity analytical ultracentrifugation (MW-SV-AUC) provides accurate extinction coefficient of AAV full and empty particles, with which quantitative size distribution analysis can be possible. Band sedimentation AUC also provide size distribution of AAV particles, with approximately 1/25-1/50 smaller sample amount than SV-AUC.

When the percentage of full particle becomes high like more than 95% full/empty ratio, cryo-electron microscopy combined with deep learning classification of the particle images is a reliable approach. Comparison of several methods for full empty ratio determination will be presented.

Capillary gel electrophoresis is one of the best approaches for VP1/VP2/VP3 stoichiometry determination. Variation of VP stoichiometry in capsid assembly results in more than two types of full particle that gives multiple bands in cesium chloride density gradient ultracentrifugation. Mass spectrometry provides detailed information on the primary structure, post-translational modification, higher order structures and dynamics of AAV particles. I'll introduce recent our study on the glycosylation analysis of AAV6. How each property has impact on the biological activity will be also demonstrated.

Based on the quantitative and detail characterizations of AAV vectors we have established, together with information from previous studies, I'd like to discuss about the best practice and future direction for AAV drug product characterization and in process analysis.

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P0236

Real-Time tracking of AAV gene therapy via MR imaging

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A new era for the gene therapy field has arrived. The number of clinical trials worldwide exceeded 2400 in 2023 and is increasing significantly. Ensuring effective clinical trials with shortened paths to approval will require robust biomarkers to track therapeutic effects. Currently, it is impossible to determine the therapeutic distribution of the gene and its product in the target tissues. This is a significant hindrance, leaving investigators guessing which organs or structures are effectively treated. Due to the lag time associated with clinical disease progression, this limitation ultimately impacts the evolution of treatment modalities.

We have developed a unique magnetic resonance (MR) imaging technology that is positioned to transform the field of gene therapy by providing real-time, non-invasive monitoring of successful gene expression in vivo. The gene therapy field lacks disease-specific and robust biomarkers by which it can track therapeutic effects. We have developed a platform of MR imaging contrast agents that are conditionally activated by the product(s) of gene therapy *in vivo*.

The need for non-invasive, disease-specific biomarkers that reflect treatment efficacy is paramount and not limited to gene therapy. Still, it applies to anything that augments the targeted enzyme activity (including enzyme replacement therapy (ERT), transcriptional read-through agents, and chaperone therapies. Here, we describe the development of a new MRI-based technology to track enzymatic activity enhancement in any organ, peripheral nervous system (PNS), or central nervous system (CNS) over time, and thus, it has the potential to play a critical role in the development and refinement of treatments for *many monogenetic enzymatic deficiencies*. Since these agents are specific to the disease, not the therapeutic modality, advancing this technology applies to the entire lysosomal disease field.

P0238

AAV Serology ELISA for testing pre-existing Immunity to AAV

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Recombinant adeno-associated virus (rAAV) vectors are leading tools for viral gene therapy. Depending on socio-demographic factors, many humans in the general population have developed antibodies against AAV as a result of naturally acquired infections. However, presence of these antibodies might directly affect efficiency and safety of the gene transfer using rAAV vectors. Furthermore, antibody levels after AAV gene therapy transfer might differ compared to those after naturally acquired infections. Thus, testing for pre-existing AAV antibodies in patient sera is indispensable for the safety of the patients, while monitoring the antibody levels after the gene transfer and comparison to the AAV antibody levels present in the healthy population will provide comprehensive data on the natural history of AAV infections as well as the immunogenicity in patients after the gene transfer.

Currently, PROGEN is in the development of AAV serology ELISAs for the most commonly used AAV serotypes (AAV2, AAV5, AAV6, AAV8 and AAV9). These assays offer a fast, sensitive and reproducible method for the quantification of total IgG antibodies against AAV in human sera. The assays contain recombinant human chimeric antibodies to generate a standard curve, making the assays reliable and reproducible.

Here, we show assay data on the limit of detection and limit of quantification, as well as linearity and reproducibility for our newly developed AAV2, 5, 6, 8 and 9 serology ELISAs.

P0239

Aligning bioanalytical and diagnostic strategies for effective patient pre-screening in AAV gene therapy trials

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Recombinant adeno-associated virus (rAAV) vectors are the leading delivery vehicle for in vivo gene therapies (GT). However, up to 70% of patients have pre-existing antibodies to AAV capsids, potentially excluding them from trials to ensure treatment efficacy and safety. The clinical relevance of pre-enrollment criteria and specific cut-off values for these antibodies has not been well-demonstrated, potentially excluding high-risk patients from beneficial treatments.

This presentation will explore the necessity of establishing clinically relevant exclusion criteria for rAAV-based GT, highlighting the need for alignment between bioanalytical and diagnostic fields. Current approaches for determining enrollment cut-offs and setting meaningful clinical decision limits (CDLs) will be reviewed. We will compare bioanalytical cut points, derived from guidelines for therapeutic protein-based drugs, with diagnostic reference intervals and CDLs.

We will discuss the unique aspects of anti-AAV antibodies as diagnostic analytes, contrasting them with classical diagnostic markers. Additionally, the differences between companion diagnostics (CDx), complementary diagnostics, in vitro diagnostics (IVD), and laboratory-developed tests (LDT) will be examined, particularly in the context of current regulatory requirements and future developments.

The session will also cover the development and optimization of anti-AAV antibody assays from preclinical stages to post-market authorization, and the potential for a single CDx per AAV serotype. Emphasizing the value of collaboration between bioanalytical and diagnostic laboratories, we aim to foster better communication across scientific fields. A glossary of key terms will be provided to bridge the language gap between diagnostic and bioanalytical disciplines.

P0240

Novel dual-plasmid system: next-generation AAV production strategy with high productivity and high quality

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The traditional triple-plasmid transfection system has been widely employed in industrial AAV manufacture due to its stable scalability and flexibility. Approximately two decades ago, a dual-plasmid system, consolidating RepCap and adenovirus helper genes into a single plasmid, was developed to reduce the cost of GMP-level plasmid manufacture and simplify the process. However, challenges such as relatively low productivity, quality issues, and the risk of replication-competent AAV (rcAAV) persist in the dual-plasmid system. In this report, we present a novel dual-plasmid system featuring a uniquely designed TP vector with optimized sequences and arrangement of vector elements. Our findings indicate that this novel system demonstrates higher AAV productivity and quality compared to the traditional triple-plasmid system, with minimal risk of rcAAV contamination. For process development, we screened seven different transfection reagents and identified a low-cost option suitable for high-titer production in the dual-plasmid system. Transfection process parameters have also been optimized to enhance productivity and reduce process-related impurities. To assess scalability, we tested the system with various therapeutic Gene of Interest (GOI) plasmids on both Ambr 250 bioreactor and 3L stirred bioreactor, demonstrating either equal or higher yields and lower process-related impurities for the dual-plasmid system. In conclusion, we have developed a next-generation dual-plasmid AAV production system that establishes a new manufacturing process platform with high productivity, quality, and cost-effectiveness.

P0241

Anti-AAV antibody persistence following gene therapy with scAAV8-LP1 hFIXco in participants with haemophilia B: A 10 year follow up

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Gene therapy for haemophilia B (HB) using adeno-associated viral (AAV) vectors encoding a functional Factor-IX gene is now approved, marking a significant milestone. Following systemic AAV vector administration, anti-AAV antibodies increase. Although this rise has no immediate clinical consequences, high levels of neutralising anti-AAV antibodies (NAB) can prevent successful future gene transfer using the same serotype. This becomes problematic if transgene expression falls below therapeutic levels and repeat dosing is required. Additionally, NABs against one AAV serotype can cross-react with other serotypes, limiting the use of alternative vectors. This study presents a 10-year longitudinal analysis of anti-AAV antibody response in severe HB participants following systemic administration of scAAV2/8-LP1-hFIXco from the St Jude-UCL Phase I/II gene therapy trial (AGT4HB ClinicalTrials.gov:NCT00979238).

Anti-AAV antibody levels were measured in 10 trial participants after an intravenous infusion of scAAV2/8-LP1-hFIXco at doses of 2x10^11 vg/kg (N=2), 6x10^11 vg/kg (N=2), or 2x10^12 vg/kg (N=6). Total anti-AAV antibodies (TAB) were assessed using ELISA, quantified with a pooled IgG reference. NABs were quantified using an in-vitro transduction inhibition assay (TIA) with AAV CMV NanoLuc on HEK 293T cells. Results were normalized, and the 50% inhibition dilution (IC50) was calculated. Anti-AAV antibody titers in 38 normal plasma samples were also evaluated for comparison.

The median follow-up was 10.7 years (range: 4-12 years), two participants were followed for only 4 years. The AAV8 TAB response showed dose dependence: low-dose participants had 84±101 mg/ml at 1 year, mid-dose participants had 507 mg/ml, and high-dose participants had 1650±603 mg/ml, representing a >1400-fold increase from pre-AAV levels, far exceeding natural infection levels (range 0.31-34.02 mg/ml). High-dose cohort TAB titers against AAV3B and AAV5 at 1 year were 145±167 mg/ml (natural range: 0.31-29.61 mg/ml) and 96±55 mg/ml (natural range: 0.31-36.13 mg/ml), respectively. TAB levels declined by year 5, with high-dose cohort levels at 928.9±556.9 mg/ml for AAV8, 90.4±95.6 mg/ml for AAV3B, and 72.8±49.1 mg/ml for AAV5. AAV8 NAB titers followed a similar trend, with IC50 values at 1 year of 7261.5±9812 (low-dose), 15000 (mid-dose), and 40204±37823 (high-dose). NAB levels declined to an IC50 of 12907±8246 at 5 years in the high-dose cohort. Cross-reactive NAB development against AAV3B and AAV5 was modest and within the range of naturally seropositive individuals.

AAV8 NAB titers followed a similar trend with IC50 at 1 year being 7261.5±9812 (low-dose), 15000 (mid-dose), and 40204±37823 (high-dose), significantly higher than natural infection levels (5-747) (Mann Whitney $p<0.0001$). NAB levels in the high-dose cohort declined to an IC50 of 12907±8246 at 5 years, still 3000-fold higher than baseline (5-70.1) (Mann Whitney $p<0.0001$) and above the threshold for efficient gene transfer. Cross-reactive NAB levels were within the range of naturally seropositive individuals: for the high-dose cohort, NAB to AAV3B at year 1 was 6536±1869 (natural range 5-13502), and to AAV5 was 852±596 (natural range 5-2143).

There is a dose-dependent rise in anti-AAV8 antibody levels that decline over time but preclude re-administration of the same serotype. Cross-reactive antibodies to other serotypes initially increase but also decline with time, potentially permitting subsequent re-administration.

P0242

Clonal analysis of ex vivo expanded mobilized peripheral blood hematopoietic stem and progenitor cells in xenografts

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Ex vivo expansion of HSPC from postnatal sources is an unmet need in gene therapy and regenerative medicine, to compensate for HSPC loss during manufacturing, to enable *ex vivo* gene therapy for young infants where leukapheresis is unfeasible & large volume bone marrow (BM) harvest is unsafe and to enable more complex genetic engineering such as gene editing & selection of engineered cells. We set out to optimize small molecule-based expansion protocols for genetically-engineered mPB HSPC employing in-depth characterization at single cell/clonal level coupled to functional in vivo readouts using xenograft models. Compounds repressing the LSD1/CoREST histone eraser complex had the strongest impact on maintaining HSC during *ex vivo* culture. The addition of an aryl hydrocarbon receptor antagonist moderately increased HSC content, although the extent depended on the culture media used. Of note, lentiviral (LV) transduction was not completely neutral, even when purified vectors were used. When combined with sphingolipid modulators, potent molecules to expand cord blood HSC, LV transduction antagonized the maintenance/expansion of primitive cells. By optimizing the choice of media, cytokines, small molecules and the timing of transduction, we were able to neutralize the negative effects of the genetic engineering step. We evaluated the clonality of expanded (EX) vs minimally manipulated (MM) HSPCs grafts. mPB $CD34^+$ HSPC were transduced with lentiviral vectors (including a barcoded LV) yielding on average two copies per cell. Part of the culture was xenotransplanted within 14 hours from transduction (MM), while the other part was expanded for 7 days in the presence of an LSD1/CoREST repressor before transplantation (EX). Human $CD45^+$ engraftment at 12 and 16 weeks was similar between MM and EX when the same starting cell dose equivalent was transplanted. Clonality was evaluated by 2 independent methods, namely LV barcode analysis and integration site analysis. Both approaches yielded similar results, showing that expanded products maintain a polyclonal structure without clonal skewing. By assessing the clonal sharing across mice within the MM and EX groups we observed a higher rate in the expanded group compared to MM group, providing molecular proof for symmetric HSC division in culture. We also assessed barcodes and IS in primary and secondary recipients as markers for clonal dynamics during serial transplantation. We observed a common pattern for both MM and EX groups. Major clones contributing to hematopoiesis in the secondary mice were often minor clones in the primary grafts, and vice versa, suggesting that a return to quiescence after HSC division preserves long-term repopulating potential. We also performed bulkRNA seq and scRNAseq analyses that provided biological insights regarding the efficacy of optimization settings and the maintenance of HSC & hematopoietic repopulation of the EX protocol. These novel readouts will facilitate the development of improved expansion protocols. In conclusion, we present a revisited expansion protocol based on clinically-validated compounds and tailored to

genetically-engineered mPB HSC, towards harnessing the full potential of ex vivo HSC expansion in the gene therapy context.

P0243

Development of precision gene engineered B cells as a treatment for hemophilia B

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Hemophilia B is an X-linked recessive bleeding disorder that affects approximately 1:20,000 males. It is caused by mutations in the *F9* gene that encodes for the factor IX (FIX) protein, an essential enzyme in the coagulation cascade. Despite advances in treatment options for hemophilia B, significant unmet needs remain, notably disease and treatment burden. Terminally differentiated human plasma cells derived from genetically engineered B cells (B Cell Medicines, BCMs), offer decade long natural longevity, capacity for high levels of protein secretion, ability to engraft without preconditioning, and are re-dosable, thus making them an attractive platform to provide durable protein replacement therapy in adults, as well as children. BE-101 is an investigational autologous B cell-derived *ex vivo* gene edited cell therapy comprised of expanded and differentiated B lymphocyte lineage cells that have been genetically engineered to express and secrete FIX. BE-101 is in development as a potential treatment for hemophilia B. In this study, primary human B cells were isolated, activated, and engineered by CRISPR/Cas9 genome editing followed by AAV-mediated homology directed repair (HDR) insertion of human *F9* gene (Padua variant) into the C-C chemokine receptor type 5 (CCR5) safe harbor locus. The cells were then further expanded and differentiated towards the plasma cell lineage, resulting in FIX-producing BCMs. Engineered BCMs secreted up to 60 ng/1e6 cells/hour of FIX protein. Vitamin K-dependent activated partial thromboplastin time (aPTT) using the one stage clotting and immunocapture chromogenic assays were employed to verify biological activity of BCM-produced FIX. FIX-expressing BCMs were transferred into immunodeficient NOG-hIL6 mice, with FIX production demonstrated *in vivo* across multiple different lots of BE-101. A single intravenous dose of BE-101 resulted in sustained hFIX levels in plasma up to 28 weeks. Biodistribution studies using a human *Alu* gene family qPCR assay confirmed rapid and sustained bone marrow engraftment with clearance from non-target/homing tissues. A robust nonclinical program of studies (including GLP toxicology and genome safety studies) has confirmed BE-101's mechanism of action and demonstrated a favorable safety profile, with no BE-101 related safety findings across any of the *in vivo* studies. Furthermore, a second dose of BE-101 at 7 weeks post-infusion resulted in an additive increase in FIX levels, demonstrating potential for re-dosability. Genotox assessments of off-target editing, and genome integrity did not identify issues of concern. In summary, we have developed an *ex vivo* precision gene engineered B cell medicine, BE-101, that produces continuous and durable levels of active FIX *in vivo*. A robust nonclinical data package of BE-101 including pharmacology, biodistribution, and safety was generated to support the proposed BE-101 first-in-human (FIH) clinical trial. US FDA cleared IND Application for BE-101. We are on track to initiate the Phase 1/2 first-in-human clinical trial in 2024.

P0244

Homology-independent targeted integration in a humanised mouse model of Haemophilia A

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Haemophilia A (HA) is the most common X-linked bleeding disorder caused by lack of factor VIII (FVIII), an essential cofactor of the coagulation cascade. Almost half of the severe HA cases are due to an inversion within intron 22 of the *F8* gene, which results in the inversion and translocation of the first 22 exons and consequent absence of FVIII expression and activity. In order to correct this common *F8* inversion by genome editing, we generated a humanised mouse model missing *F8* exons 23 to 26 and instead carrying the first 4 kilo bases of the human *F8* intron 22. We then used adeno-associated viral (AAV)-mediated homology-independent targeted integration (HITI) directed to liver to insert the last missing *F8* exons (23 to 26) downstream of the exon1-22 translocation, thus restoring FVIII expression. To do this, 2 different AAV8 vectors were used: one vector encodes *Staphylococcus pyogenes* CRISPR/Cas9 (*SpCas9*) under the expression of a liver-specific promoter; another vector encodes the HITI donor containing the mouse *F8* exons 23 to 26 flanked by two inverted gRNA sites, and the U6 promoter expressing the gRNA specific for the human *F8* intron 22. Preliminary results show positive donor integration of all gRNA-treated animals by PCR amplification of DNA extracted from liver samples. In contrast, mice administered with tested constructs show undetectable FVIII activity levels and prolonged coagulation times at various time points post-injection, regardless of the promoter used to express SpCas9. These findings suggest that the donor integration at the target site is taking place but not at sufficient levels and/or in the correct liver cell type. We are currently evaluating the platform using a strong ubiquitous promoter (full-length cytomegalovirus promoter) to express *SpCas9* and achieve stronger correction in different liver cell types, particularly in liver sinusoidal endothelial cells (LSECs), the physiological producers of FVIII. Restoration of FVIII expression in a relevant portion of the LSEC population could result in therapeutic efficacy in HA mice.

P0245

Investigating the role of ADAMTS18 in angiogenesis

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A Disintegrant and Metalloproteinase with Thrombospondin motif (ADAMTS) family includes 19 zinc-metalloproteinases that are proteolytically active in a variety of physiological processes, particularly in extracellular matrix remodelling. ADAMTS enzymes have also been linked to the pathogenesis of different diseases, such as vascular pathologies, arthritis and cancer. First

identified in 2002, ADAMTS18 is an orphan member of the ADAMTS family which has been detected in several fetal and adult tissues, including brain, heart and endothelium. Furthermore, *Adamts18* knockout mice have demonstrated a role for ADAMTS18 in lung, kidney and eye development, by affecting epithelial branching. Whereas the mechanisms of its involvement in different diseases, including cancer, bone- and eye-related diseases remain largely unknown. In the last few years, a role for ADAMTS18 in early vascular development and adult angiogenesis has been unravelling. Recently, our group reported that ADAMTS18 downregulation impairs endothelial tube formation in primary endothelial cells as siRNA knockdown of *ADAMTS18* in HUVEC resulted in a remarkable reduction in the sprout area and length. ADAMTS18 deficiency has also been shown to impair trunk angiogenesis and decrease the expression of angiogenesis related genes in zebrafish embryos. Furthermore, *Adamts18* knockout was shown to affect early development of the embryonic aortic artery and the common carotid artery in mice. These findings suggest a potential involvement of ADAMTS18 in angiogenesis and understanding of the mechanisms of its action may pave the way for its use in angiogenic gene therapy. Hence, our aim is to investigate the role of ADAMTS18 in angiogenesis. To do this we have overexpressed ADAMTS18 in primary endothelial cells and animal models, using a lentiviral vector that we developed, and observed its effects on cellular proliferation, adhesion and tube formation. Our results demonstrate that ADAMTS18 overexpression has variable effects on these endothelial cell processes and ongoing testing is delineating the mechanisms of ADAMTS18 activity.

P0246

Novel lentiviral vectors for the treatment of acute myocardial infarction

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Improved prevention and treatment strategies of acute myocardial infarction led to a substantial decline in cardiovascular disease (CVD)-related mortality in almost all European countries in the last decades. However, CVDs are still the leading cause of death worldwide as well as in Europe. Over 80% of deaths are due to myocardial infarction and stroke leading to a huge economic burden. Due to diffuse chronic disease or comorbidities, up to one third of the patients are not suitable for current treatments. Therefore, novel therapeutic approaches are needed to treat CVD and increase long term survival in cardiac ischemia patients. Recently, gene therapy has been celebrating clinical successes, but applications targeting the heart have been scarce due to various reasons. Thus, our aim was to develop a lentiviral vector to stimulate blood vessel growth by therapeutic angiogenesis in order to increase the tissue perfusion and thus support tissue regeneration and recovery after myocardial infarction.

Here we use endothelial specific lentiviral vectors expressing Vascular Endothelial Growth Factor Receptor 2 (VEGFR2) and C-X-C Motif Chemokine Receptor 4 (CXCR4) in a mouse model of acute myocardial ischemia. Myocardial infarction is induced in adult C57Bl/6J mice by ligating the left main descending coronary artery. Gene transfer of lentiviral vectors were performed intramyocardial after successful ligation. Cardiac performance was accessed by echocardiography before surgery and 7 days after myocardial infarction and gene transfer.

We could show that mice treated with endothelial specific lentiviral vectors expressing VEGFR2 had significantly decreased cardiac performance compared to baseline results. This finding is also

reflected by impaired survival. Mice treated with a lentiviral vector containing CXCR4 resulted in improved survival as compared to VEGFR2 as well as preserved cardiac performance 7 days after infarction. These results demonstrate that an angiogenic vector is not beneficial in treatment of myocardial infarction and further studies are ongoing with CXCR4.

P0247

Pre-clinical development of gene augmentation therapy to restore Acetyl Cholinesterase activity in Collagen Q Congenital Myasthenic Syndrome using a platform approach to AAV gene therapy development

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Amplo Biotechnology is developing AMP-201 (AAV-ColQ) to augment Collagen Q (ColQ) in skeletal muscle. ColQ deficiency leads to impaired Acetyl Cholinesterase anchoring and abnormal neuromuscular junction (NMJ) formation in a condition known as ColQ Congenital Myasthenic Syndrome (CMS). ColQ patients have severe defects in the neuromuscular transmission that lead to neuromuscular failure. Given the rarity of ColQ CMS (~1,000 patients in the major economies) and its similarity to other CMS (e.g., Dok-7 CMS), we applied a platform approach to decrease the costs of development and time to the clinic. In this study, we report positive results from our pharmacology study and small-scale suspension manufacturing, and we will describe how we plan to leverage AMP-101's (AAV Dok-7) development for AMP-201.

P0248

Improving cardiac specific promoters for AAV gene therapy

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Gene therapy has revolutionized our capacity to treat and potentially cure not only rare genetic diseases but also common diseases such as cardiovascular disease. In the cardiac field, Adeno-Associated Virus (AAVs) has been used for almost three decades to deliver therapeutic genes due to the safety and efficiency provided by these vectors. However, no cardiac gene therapies have been approved to date, as significant clinical improvements after treatment thus far have been limited. To enhance the effectiveness of gene therapy in the heart, vector optimizations are required to achieve therapeutic efficacy-threshold levels of gene expression. In this study, we have focused on heart-specific promoter optimization, by designing promoters that could drive high levels of cardiac expression and specificity. To achieve this, we combined the enhancer regions of two cardiac specific promoters (CE1,CE2), one skeletal muscle promoter (ME1), and a strong ubiquitous promoter (UE) with the core promoters of a cardiac specific promoter (CCP), an

ubiquitous core promoter selected for strength (UCP) and a complete skeletal muscle promoter (SMP). A total of twenty-two different promoters were designed and their expression levels were analyzed in Neonatal Rat Ventricular Cardiomyocytes (NRVMs), and mouse cell lines C2C12 myoblasts and HEPG2 hepatocytes. We compared our promoters' strength and specificity to drive reporter expression with that of the cytomegalovirus (CMV) promoter and of the human cardiac troponin 2 (*TNNT2*) promoter, the current standard to drive cardiac-specific expression in AAV vectors. Our in vitro results lead to the identification of two interesting candidate promoters. Our first candidate, CE1-CCP, is 250 bp shorter than the *TNNT2* promoter, has shown expression levels akin to *TNNT2* in NRVMs, and has a parallel degree of specificity with a similar low expression in the main off target cell lines. Interestingly, a second candidate promoter, CE1-SMP, displayed levels of expression similar to that of the CMV promoter in heart, while showing a ~ 66% higher expression than the CMV promoter in skeletal muscle. Its activity level in liver cells was below the negative control, which contained only the inverted terminal repeats, suggesting a potential use in diseases where all muscle tissue must be targeted, which warrants further investigation.

P0249

Generation of cardiomyocyte-specific Snhg5 knockout mice by CRISPR/Cas9

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To analyze the function of endogenous Snhg5 in the heart, we established cardiomyocyte-specific Snhg5 knockout mice through Crispr/Cas9 technology. Edu incorporation experiments on neonatal mouse cardiomyocytes confirmed that knocking out Snhg5 inhibited cardiomyocyte proliferation. In vitro and in vivo overexpression experiments demonstrated that Snhg5 can promote the proliferation of mouse cardiomyocytes. To evaluate whether Snhg5 was also involved in the repair process of cardiac damage by regulating cardiomyocyte proliferation, we established a myocardial infarction (MI) model by performing coronary artery ligation surgery on cardiomyocyte-specific Snhg5 transgenic mice. By conducting echocardiography analysis before myocardial infarction and 1 week, 3 weeks, 4 weeks and 5 weeks after MI, it was found that Snhg5 overexpression can partially improve myocardial contractile dysfunction caused by myocardial infarction surgery and promote the proliferation of cardiomyocytes in the border and remote regions. This suggests that overexpression of Snhg5 can protect the heart from MI-induced damage by promoting cardiomyocyte proliferation and cardiac repair. The above research results indicate that Snhg5 plays an important role in regulating myocardial cell proliferation and regeneration.

P0250

The secretome released by IFN-γ-primed cardioesphere-derived cells as a therapeutic alternative in the treatment of myocardial infarction

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Restoration of coronary blood flow following myocardial infarction (MI) is facilitated by thrombolytic drugs. These post-MI therapies aim to prevent complications such as heart failure and arrhythmias. Innovative treatments, such as the secretome released by cardioesphere-derived cells (CDCs), have shown promising results in cardiac tissue regeneration and arrhythmia prevention. In this study, we investigate the potential of inflammatory priming of CDCs to enhance therapeutic outcomes.

To assess the impact of secretome from primed CDCs (S-pCDCs) on MI treatment, we conducted an in silico analysis focusing on the efficacy of specific medications, including Captopril (antihypertensive), Bisoprolol (antihypertensive), and Spironolactone (beta-blocker).

CDCs (n=4) were isolated from cardiac explants of Large White pigs and cultured in DMEM + 1% ITS, antibiotics, and IFN-γ (3ng/ml) for 72 hours. The resulting conditioned medium was centrifuged and ultrafiltered (3KDa) to obtain S-pCDCs. Subsequently, the content of S-pCDCs (miRNA and transcripts) was analyzed using transcriptomic techniques and qPCR.

Bioinformatic tools were used to first obtain the interaction network (IN) of the three mentioned drugs with their target genes, through which they manifest their therapeutic effects, using the String App. Then, the web tool miRTargetLink 2.0 was used to reveal the miRNAs interacting with the genes of interest. Similarly, the target genes of these aforementioned genes were identified using the FunRich analysis tool. Finally, the content of S-pCDCs was compared with the previously identified miRNAs and genes, and an IN was generated using Cytoscape.

Our results revealed that S-pCDCs contain miRNAs that interact with the target genes of the drugs. Six correspond to the target genes of Captopril, and one to Spironolactone. Additionally, S-pCDCs contain transcripts that match those affected by the drugs, predominantly by Spironolactone.

This analysis suggest that the treatment with S-pCDCs could mimic the effects of Captopril and Spironolactone, simplifying MI treatment. However, further validation studies are required to confirm these findings.

P0251

Adeno-associated viral (AAV) mediated gene replacement therapy for SLC25A4 deficiency

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Nuclear DNA-encoded mitochondrial proteins that harbor mutations can impair oxidative phosphorylation and are thus linked to the development of skeletal myopathy, cardiomyopathy, and cardiac failure. We previously studied a 13-generation Mennonite pedigree with autosomal recessive skeletal myopathy and hypertrophic cardiomyopathy due to a frameshift mutation in solute carrier family 25, member 4 (*SLC25A4*; c.523delC, p.Q175RfxX38). *SLC25A4* encodes the heart-muscle isoform of the adenine nucleotide translocator-1 (SLC25A4), which is an important member of mitochondrial oxidative phosphorylation. The single base pair deletion in *SLC25A4* prematurely terminates translation of the SLC25A4 protein; the loss of function of this protein thus results in dysfunctional mitochondrial metabolism. Affected individuals display exertional intolerance, hyperalaninemia, lactic acidosis, and persistent adrenergic activation. Cardiac dysfunction and myocardial thickening ultimately progress to end-stage heart failure necessitating cardiac transplantation. We hypothesize that restoration of SLC25A4 activity through recombinant adeno-associated viral (rAAV) delivery can be a disease-modifying therapy for SLC25A4 deficiency, resulting in restoration of myocyte cell bioenergetics, correction of biochemical abnormalities, and improvement in skeletal muscle and cardiac function. A cross-species evolved, liver-detargeted AAV capsid packaging the *SLC25A4* transgene under the control of a cardiac/musculoskeletal promoter was generated. When this rAAV-SLC25A4 vector is delivered to human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs) and mouse myoblasts *in vitro*, SLC25A4 is expressed and appropriately localizes to the inner mitochondrial membrane. Preliminary assessment of mitochondrial functional rescue using the Seahorse assay will be presented. We next generated and characterized a *Slc25a4* knockout mouse (Slc25a4-KO) model. By 14 weeks of age, Slc25a4-KO mice have plasma alanine levels 1.8 times higher than age-matched control mice (p=0.022). Slc25a4-KO mice develop a concentric dilated cardiomyopathy characterized by substantial myocardial hypertrophy and ventricular dilation, with progressive cardiac dysfunction (p=0.018). Systemic delivery of rAAV-SLC25A4 to Slc25a4-KO mice *in vivo* resulted in effective transgene delivery and robust SLC25A4 protein expression in cardiac and skeletal muscle tissue, with reduced transgene biodistribution and expression in off-target organs. We will present 6-month functional skeletal muscle and cardiac data following treatment with therapeutic AAV-SLC25A4. AAV-mediated gene replacement may offer a disease-modifying therapeutic strategy for treatment of SLC25A4 deficiency.

P0252

A novel AAV gene therapy for treatment of BAG3 dilated cardiomyopathy

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Gene therapy with adeno-associated virus (AAV) vectors is emerging as a promising therapeutic platform for the treatment of cardiovascular disease. Unfortunately, effective gene delivery to the heart and safety concerns have been major limitations to clinical translation. Achieving therapeutically relevant levels of cardiomyocyte transduction with IV delivered AAV vectors at doses that are well tolerated has been the field's most pressing challenge. We have identified novel AAV capsids by a machine learning-guided rational design approach that demonstrates significantly improved cardiac transduction at lower doses compared with wild-type AAV9.

Dilated cardiomyopathy (DCM) is defined as dilation and systolic impairment of the left ventricle and is the most common cause of heart failure (HF) in the young and most frequent indication for heart transplantation. ≥50% of patients with DCM have a genetic predisposition to the disease. BAG3 which encodes for B-cell lymphoma 2 (Bcl-2) associated anthanogene-3 (BAG3), a cochaperone that interacts with members of the heat shock protein (HSP) family, has been identified as a DCM-causing gene. BAG3-DCM represents a significant unmet medical need in a patient population with rapidly progressive cardiac dysfunction for whom no treatments targeting the underlying mechanism of disease exist.

We are developing an AAV-mediated gene replacement strategy to treat BAG3 DCM using a cardiotropic capsid with an improved safety profile and report therapeutic benefit in two mouse models of cardiomyopathy.

To evaluate therapeutic efficacy in a disease-relevant context, we employed both a surgically induced mouse model of myocardial infarction (MI) as well as a conditional BAG3 KO mouse with cardiac-specific haploinsufficiency (cBAG3+/−). Mice received IV injection of AAV expressing human BAG3 under the control of a cardiac promoter at a timepoint in which both mouse models exhibit significant cardiac dysfunction as measured by echocardiography. Based on biodistribution studies performed in wild type mice we selected an IV doses that led to >70% transduction of the mouse myocardium. We demonstrate that both myocardial infarcted mice and BAG3 cKO mice show significant improvement in cardiac function (ejection fraction, fractional shortening, LVIDs, LVIDd) following systemic injection of our vector. Histological analysis demonstrated no evidence of test article-related safety findings in any tissue analyzed (heart, skeletal muscle and liver). Immunohistochemistry confirmed BAG3 expression in up to 70% of the myocardium and expression throughout the heart with little to no expression in skeletal muscle.

In summary, we propose an AAV-mediated gene replacement using a cardiotropic capsid as a clinically viable approach to achieve broad and therapeutic levels of BAG3 in the heart while detargeting off target tissues.

P0253

CRISPR/hfCas12Max-based single-cut gene-editing therapy restores dystrophin expression and muscle performance in mouse models of Duchenne muscular dystrophy to support the initiation of MUSCLE clinical trial

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Duchenne muscular dystrophy (DMD) is an X-linked, progressive muscle disease due to the pathogenic variants in the *DMD* gene encoding dystrophin. The antisense oligonucleotide medicines, known as Eteplirsen or Casimersen, have been approved to treat DMD patients with exon 45–55 hotspot region mutations. However, it has been shown that the Eteplirsen or Casimersen restores dystrophin protein expression <1% of the normal level after one year of continuous administration. Here, we developed a single administration of CRISPR/high-fidelity(hf)Cas12Max-mediated single-cut gene-editing therapy for DMD. Two humanized DMD mouse models were generated; one replaced mouse exon 51 with human exon 51 and deleted mouse exon 52 ($DMD^{\Delta mE51E52,hE51KI}$), while the other replaced mouse exon 45 with human exon 45 and deleted mouse exon 46, thereby disrupting the open reading frame of the dystrophin protein. We examined the editing efficiency of hfCas12Max in human *DMD* exon 51 splice donor (SD) or splice acceptor (SA) sites and the SD of exon 45 *in vitro*. A single adeno-associated virus (AAV) vector packaged with hfCas12Max (AAV-hfCas12Max) and a guide RNA (gRNA) targeting the SD or SA sites of human exon 51 or 45 was injected into the DMD mice and wildtype non-human primates (NHPs) to evaluate the *in vivo* editing efficiency, dystrophin expression, and muscle functions. Both humanized DMD mouse models exhibited phenotypes highly similar to DMD patients, suggesting they are suitable disease models. Then, we plan to initiate the **MUSCLE** clinical trial (an open-label, **M**ultidose dose-escalation study to **U**nderstand the **S**afety of **C**RISPR gene-editing therapy and its long-**L**asting **E**ffects in DMD patients). Our findings showed that hfCas12Max-mediated single-cut gene-editing efficiently targeted the SD sites of human *DMD* exons 51 and 45 *in vitro*, achieving an editing efficiency of over 60%. Systemic delivery of AAV-hfCas12Max-exon51 (HG302) effectively restored dystrophin protein expression in these two DMD mouse models and improved the histopathology and grip strength of these DMD mouse models. Additionally, HG302 induced therapeutically acceptable editing efficiency of exon 51 SD in healthy wild-type NHP at low dose with no observed hfCas12max-related toxicity, suggesting its potential good safety profile. Based on these preclinical data, our first-in-human **MUSCLE** trial will enroll 4-6 ambulant boys aged 4 years or older with confirmed deletion of exon 52, 52-61, or 52-63 with measurably impaired muscle function (defined as <80% of the predicted value). Besides the safety assessments, the efficacy objectives include the change from baseline in the percentage of dystrophin-positive fibers and dystrophin expression intensity, skeletal muscle dystrophin expression, and several functional ambulation assessments such as the North Star Ambulatory Assessment (NSAA). Our novel discovery of hfCas12Max nuclease and rigorously designed and executed *in vitro* and *in vivo* tests support the clinical development of an 'all-in-one' AAV with the CRISPR/hfCas12Max gene-editing therapy for DMD patients. The U.S. FDA has granted orphan drug and rare pediatric disease designations to HG302 for treating DMD.

P0254

Repurposing CRISPR/Cas13 systems for therapeutic mRNA trans-splicing in cardiac and musculoskeletal disorders

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The cellular RNA processing machinery has many attributes that can be exploited to manipulate the transcriptome. Specifically, RNA splicing is well conserved in higher eukaryotes and has been leveraged to design therapeutic approaches for a number of diseases. Here, we introduce CRISPR Assisted Fragment Trans-splicing (CRAFT), a novel RNA editing approach that repurposes CRISPR-Cas13 systems to promote trans-splicing of recombinant RNA fragments into a target endogenous pre-mRNA transcript. This platform leverages the endogenous RNA splicing machinery to precisely rewrite multi-kilobase mRNA stretches and is agnostic to mutation composition. These unique characteristics of CRAFT enable uniform RNA correction across a diverse mutation spectrum with a single editing strategy. We present a robust evaluation of the CRAFT approach for editing RNA transcripts associated with different muscular dystrophies (*DMD* and *DMPK*) as well as cardiomyopathies (*LMNA*, *FXN*, and *RYR2*). Specifically, we demonstrate CRAFT mediated editing in fibroblasts isolated from Hutchinson-Gilford Progeria Syndrome patients, a gain of function laminopathy. In addition, we have developed a barcode-based screen to rapidly optimize key parameters associated with guide design for targeting different intronic regions using CRAFT. We envision that CRAFT may be an attractive platform for treating disorders characterized by toxic gain-of-function (GOF) mutations or large genes with loss of function mutations (LOF).

P0255

Complete Genetic Correction of Duchenne and Becker Muscular Dystrophy Using Chromosome Transplantation in Induced Pluripotent Stem Cells

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Duchenne (DMD) and Becker Muscular Dystrophies (BMD) are X-linked disorders caused by mutations in the *dystrophin* gene (Xp21.2) encoding the dystrophin protein and leading to a progressive and irreversible muscle deterioration. DMD is determined by the complete loss of the dystrophin protein, while BMD, carrying in-frame mutations that lead to the production of a shorter form of protein with reduced functionality, exhibits a milder phenotype. Due to the huge size of *dystrophin* gene and the typical large genomic mutations associated with both clinical forms, conventional gene therapy approaches are unable to completely correct these genetic defects. To date, the only available therapeutic strategy aims at converting DMD into BMD with only a partial rescue of the disease phenotype. Here, we propose a novel genomic approach previously developed in our lab, namely Chromosome Transplantation (CT), to fully correct the molecular defect of the *dystrophin* gene in induced pluripotent stem cells (iPSCs). CT consists in the perfect substitution of an endogenous defective chromosome with an exogenous normal one, resulting in a normal euploid karyotype with a complete resolution of the gross mutation. To achieve our purpose, we exploited CT to correct both DMD- and BMD-iPSCs, and verified the functional recovery of dystrophin protein in cardiomyocytes (CMs) differentiated from both disease and corrected iPSC lines. CT is a multi-step protocol; the first step consists in the transfer of an entire normal exogenous X chromosome to DMD/BMD patient-derived iPSCs. The second

step entails the selection of cells containing the exogenous normal X chromosome. For this purpose, we took advantage of a selection system based on the X-linked *HPRT* gene. By CRISPR-Cas9 technology, we successfully inactivated the *HPRT* gene in our targeted cells (both parental BMD and DMD cells) and produced a donor cell line containing a normal X chromosome. For chromosome transfer, we used an improved microcell-mediated chromosome transfer (RETRO-MMCT) approach. Cells containing wild-type *HPRT/dystrophin* X chromosome were selected, and clones that spontaneously lost the endogenous X chromosome, resulting in a perfect substitution of the defective X chromosome, were identified. We analysed the isolated chromosome transplanted iPSCs (CT-iPSCs) for genomic stability, stemness and pluripotency capability. To confirm the success of the CT, we performed whole exome sequencing (WES), which showed the absence of unexpected different variations (DV) due to manipulation, and the presence of the expected DV linked to the transplanted X chromosome. In addition, CMs differentiated from CT-iPSCs showed the expression of the complete form of dystrophin, both at the transcriptional and protein levels, further indicating the successful correction of the genomic defect. Finally, in order to demonstrate the functional rescue, we performed a comprehensive electrophysiological analysis on CMs differentiated from both pathological and CT-iPSCs. This analysis showed the restoration of normal contraction/relaxation and Ca2+ dynamics in the corrected CMs. In conclusion, these findings demonstrate that CT is an innovative approach capable of correcting *dystrophin* gross mutations, opening the opportunity for its potential application to other X-linked genomic diseases and, using a different selection technology, to autosomal diseases as well.

P0256

Novel human heart-derived natural adeno-associated virus capsid combines cardio specificity with cardiac tropism in vivo

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Introduction: Recombinant adeno-associated viral (rAAV) vectors currently used in clinical trials for cardiac gene therapy (GT) lack myocardial specificity. rAAV9, being clinically probed in human cardiomyopathy GT trials, exerts high hepatic toxicity which resulted in fatal liver failure and death in several patients. Against this background, rAAVs with high cardiac specificity and ability to transduce the human heart would be close to an ideal vector for human cardiac GT.

Hypothesis: We therefore hypothesized that the human heart may be a source for identifying novel AAVs, therefore capable of transducing human myocardium, with appropriate cardiac specificity to enable safe and effective gene transfer to mammalian hearts in vivo.

Approach and Results: We identified AAV9 and AAV6 in myocardial biopsies from heart failure patients as serotypes known to effectively transduce myocardium in vivo and in vitro. Additionally, a group of novel AAVs (AAV-C1-7) were discovered. HEK293 high-titer producible vectors with cloned AAVC1-7 and AAV9 were first used for in vivo biodistribution analysis with a CMV-YFP reporter cassette in mice. 2 weeks after intravenous (i.v.) injection (rAAV 1E12 vgc/kg), the AAVC7 equaled rAAV9s excellent cardiac transduction and YFP mRNA expression abilities by

vgc and mRNA levels in left ventricles (LV). In contrast to rAAV9s significantly higher hepatic than cardiac enrichment, AAVC7 was almost undetectable in liver and other organs (e.g. brain) with a 1000-fold higher heart/liver vgc ratio than rAAV9. Importantly, AAVC7 induced three-fold lower levels of post-treatment anti-capsid serum antibodies than AAV9 that were non-cross reactive.

I.v. injection of AAVC7 and rAAV9 in adult farm pigs (1E12 vgc/kg), each with a CMV-YFP reporter system, confirmed the high cardiac specificity of AAVC7 over AAV9 and homogeneous LV and RV transduction of the human-sized hearts. In hiPS-CM-EHTs, the ability of AAVC7 (1E9 vgcs per EHT) to homogenously transduce a human myocardial tissue equivalent was validated using YFP protein expression analysis via microscopy. AAVC7 biodistribution was further compared with rAAV9 in mice using a cardiac troponin (cTnT)-YFP reporter system with clinically relevant dosages (5E12, 5E13, 5E14 vgc/kg). 2 weeks after i.v. injection, rAAV9 showed high dose-dependent hepatic YFP protein IHC and IB expression. In contrast, AAVC7 unveiled the desired dose-dependent homogeneous cardiac YFP protein expression in cardiomyocytes but no detectable hepatic YFP protein expression. Given its high cardiac specificity, AAVC7 with the cTNT-relaxin family peptide receptor 1 (rfxp) inotropic response gene cassette, which can be acutely activated using the peptide ligand relaxin (rel), was injected into mice (1E14 vgc/kg). After 2 weeks, AAVC7-rfxp significantly increased cardiac contractile performance by LV catheterization in response to 50 µg single i.v. rel. injection in comparison to AAVC7-luciferase control.

Conclusion: AAVC7, discovered in human failing myocardium, combines the clinically desirable properties of high and homogeneous in vivo transduction of murine and human-sized pig hearts with excellent liver detargeting after systemic injection. Such characteristics may destine AAVC7 as a suitable vector for safe and effective human cardiac GT.

P0258

Development of AAV-mediated KCNH2 gene therapy for post-myocardial infarction arrhythmias

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Cardiac arrests is still one of the leading causes of mortality worldwide. Ventricular arrhythmia associated with myocardial infarction scarring represents a prominent cause of cardiac arrest. The border zone of the infarct is typically characterized by fibrotic tissue intermingled with viable bundles of cardiomyocytes which leads to heterogeneities in conduction and repolarization. Consequently, the infarct border zone provides an important substrate for reentrant arrhythmias that can deteriorate in ventricular tachycardia (VT) and fibrillation (VF). The current treatments (including anti-arrhythmic drugs, catheter ablation, and device therapy with an implantable cardioverter defibrillator), offer no prevention and are often accompanied by off-target effects and associated with major complications. One possible strategy to prevent reentrant excitation in the VT-isthmus has been provided by local prolongation of the effective refractory period (ERP) of scarred myocardium. To this end, previous research indicated that Adenoviral (AdV) delivery of the dominant negative potassium channel variant KCNH2-G628S lengthened APD and increased local refractoriness, thereby eliminating inducible VT in the porcine chronic MI model. This mutant represents a well-characterized and promising anti-arrhythmic target; however, sustainability of these robust therapeutic outcomes was limited by the use of short-term AdV vectors. To translate this approach into an effective long-term therapy, we have continued with the development of an effective adeno associated virus (AAV) gene therapy. To overcome

the limited AAV insert capacity (of ~5kb), we prepared a truncated version, designated KCNH2-G628SΔ. The functional consequence of the truncation on APD was tested in human induced pluripotent stem cell-derived cardiomyocytes (iPSC-CMs). Lentiviral delivery of the truncated version showed similar lengthening of the APD as the full length protein, validating the efficacy of KCNH2-G628SΔ. Next we identified the most potent AAV expression cassette for KCNH2-G628SΔ expression by *in vitro* transduction of neonatal rat ventricular myocytes. Our follow-up experiments will focus on functional testing using ventricular tissue slices obtained from sub-epicardially transduced porcine myocardium and VT inducibility testing in the porcine MI model.

P0259

CRISPR prime editing strategies for modeling cardiomyopathy in native human heart tissues

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Dilated cardiomyopathy (DCM) is a cardiac disorder characterized by left ventricular or biventricular ballooning and impaired contractility. Up to a third of DCMs are thought to have a monogenic etiology. Mutations in e.g., sarcomeric, nuclear envelope, or cytoskeletal genes lead to decreased cardiac contractility and conduction defects, eventually leading to arrhythmia, heart failure, and sudden cardiac death. To date, the only curative approach is heart transplant, while etiological treatments are lacking. Mutations in the *LMNA* gene are among the most prevalent candidates associated with severe genetic cardiomyopathy. We used CRISPR prime editing (PE) to model and/or correct DCM-associated pathogenic variants in human cell lines and iPSC-derived cardiomyocytes carrying specific *LMNA* mutations. We compared different PE proteins and editing approaches to improve editing frequencies and screened pegRNAs to identify the most promising guides for correction. However, when tested in iPSC-derived cardiomyocytes, pegRNAs led to markedly different editing efficiencies from what had been observed in HEK cells, indicating that efficiencies for the same spacers can vary substantially across different cell types, even within a confined locus that is amenable to prime editing per se. We also compared adenine base editor (ABE) and PE for gene correction of the *LMNA*-R190W mutation, showing relevant bystander activity when using SpG-ABE8e that can be reduced using SpRY-ABE8e. To evaluate and optimize the delivery and editing of different CRISPR machineries into native cardiomyocytes, we switched to a more clinically relevant *ex-vivo* model system. We transfected native pig heart tissues with lipid nanoparticles (LNPs) and mRNA encoding a fluorescent reporter and observed specific signal from up to 30% of cadiomyocytes. Next, we established LNP *ex-vivo* transfection of human native tissue, showing considerable differences in LNP delivery between native human and pig tissues. After this initial *ex-vivo* LNP optimization, we are now testing various CRISPR modalities to characterize gene editing in native human heart tissues for the first time.

P0260

Lumican inhibits cardiac angiogenesis

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The mammalian heart loses its regenerative potential within the first week after birth, concomitant with a decline in its angiogenic capacity. While many studies have investigated the changes that occur in cardiac cells, and in particular in cardiomyocytes, that might be responsible for this loss of regenerative ability, little is known about the role of the extracellular matrix (ECM) in controlling cardiac regeneration and angiogenesis. Thus, our study wishes to investigate the alternations that affect cardiac ECM early after birth and assess their contribution to the loss of angiogenic potential in the post-natal heart. We profiled the proteome of vascular cells and the surrounding ECM of neonatal and adult hearts. To label proteins accessible from the cardiac vasculature *in vivo*, we perfused neonatal and adult hearts with a biotin-containing solution. The hearts were then homogenized, and biotinylated proteins were enriched using streptavidin-conjugated beads for their identification with mass spectrometry. Bioinformatic analysis provided a list of proteins differentially expressed in the vessels of neonatal and adult hearts. Lumican, a small leucine-rich proteoglycan produced by fibroblasts, emerged as significantly enriched in the adult vasculature. We observed that lumican isoforms had a higher molecular weight in the adult compared to the neonatal heart. To investigate whether the differential glycosylation of lumican in neonatal and adult heart influences the angiogenic potential of the organ, we purified lumican directly from tissues by immunoprecipitation with magnetic beads and tested its activity on angiogenesis. Either adult or neonatal lumican was incorporated into collagen gels, used as a seeding substrate for cardiac ECs. We observed that ECs seeded on adult lumican proliferated significantly less than those seeded on either neonatal lumican or pure collagen, consistent with reduced MMP-14 activity, a key target for lumican, in the adult heart. In vivo lumican knockout in mice resulted in a significant increase in both endothelial cell number and vessel density in the heart, consistent with a role of adult lumican in inhibiting cardiac angiogenesis. Injection of a siRNA targeting lumican after myocardial infarction in adult mice led to an increased rate of endothelial cell proliferation and formation of new blood vessels. Overall, our findings set lumican as a negative regulator of cardiac angiogenesis in the adult mammalian heart and pave the way to new therapeutic interventions targeting lumican and its post-translational modifications to enhance cardiac revascularization.

P0261

Exploring AAV-Mediated Gene Therapy for PLN-R14del Cardiomyopathy: Preclinical Insights and Therapeutic Potential

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The PLN-R14del mutation, identified as a founder variant from Greece and the Netherlands, often manifests with symptoms linked to dilated cardiomyopathy (DCM) and arrhythmogenic cardiomyopathy (ACM). These symptoms include low-voltage ECG, frequent ventricular arrhythmias, and progression to end-stage heart failure, typically emerging around the fourth decade of life, although some carriers may remain asymptomatic. Phospholamban (PLN) plays a crucial role in calcium regulation within cardiomyocytes by primarily inhibiting the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2a). The PLN R14del mutation is characterized by calcium handling dysregulation, metabolic dysfunction, and protein aggregation. Current treatments focus on symptom management, preventing sudden cardiac death, and heart transplantation in severe cases.

This study aimed to evaluate the effectiveness of adeno-associated virus (AAV)-mediated treatments in humanized PLN-R14del (hPLN-R14del) mice. The treatment involved overexpressing wild-type PLN, which has been shown to effectively improve calcium transient dysfunction in PLN-R14del iPSC-derived cardiomyocytes.

Three-month-old heterozygous (HET) hPLN-R14del mice were injected with $1x10^{14}$ vg/kg of the AAV9-hPLN vector or a saline control. Five weeks post-treatment, efficacy was evaluated using a stress-induced arrhythmia protocol. Baseline ECG recordings were taken for five minutes, followed by simultaneous intraperitoneal injections of epinephrine (2mg/kg) and caffeine (120 mg/kg), with recordings taken 10-15 minutes post-injection. Following treatment, mice were euthanized, and their hearts were analyzed to evaluate treatment efficiency using droplet digital PCR (ddPCR).

HET mice showed significantly increased arrhythmia susceptibility compared to wild-type mice. Notably, treatment with the AAV9-hPLN vector in HET mice led to a 20% increase in wild-type PLN expression and resulted in decreased arrhythmic events compared to saline-treated controls.

AAV-mediated gene therapy has demonstrated sufficient efficiency in promoting the overexpression of a target gene in a preclinical model of PLN-R14del cardiomyopathy and has improved arrhythmia susceptibility. These findings suggest potential treatments for PLN-R14del-associated cardiomyopathies through efficient gene expression modulation.

P0262

Engineering cell-specific CRISPR editors for human heart cells

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Cardiovascular disease (CVD) is the leading cause of morbidity and mortality worldwide. Despite the progress in CRISPR technology with improved editing capabilities and the prospect of cardiac gene therapies, a significant hurdle remains in achieving precise and efficient editing across clinically relevant post-mitotic cells, such as human cardiomyocytes. While some gene therapies have been developed for cell or tissue-specific expression, current CRISPR editors lack bespoke variants for specific cell types that are adapted to distinct regulation and activity of DNA damage response and repair pathways. We propose engineering CRISPR editors optimized specifically for heart cells, informed by transcriptomic analysis of DNA repair in these cells. Our method includes screening candidate DNA repair genes to assess their impact on different types of CRISPR gene editing. 28 candidates were identified through RNA-seq analysis comparing cardiomyocyte transcriptome profiles at various maturation stages. To confirm and refine the specificity of our findings to cardiomyocytes, we also perform parallel assessments of these candidates in a non-cardiac cell line. Preliminary studies suggest the editing outcome alters differently in iPSC-derived cardiomyocytes (iPSC-CMs) than in HEK-293T when the 28 candidates are co-expressed with the CRISPR editor. More precisely, we saw none of the candidate DNA repair genes significantly alter the editing efficiency when co-expressed with SpCas9-nuclease in HEK-293T, whereas, in iPSC-CMs, this leads to significant fluctuations, underlining the cardiomyocyte distinctive nature of our study. On the other hand, editing with SpCas9-PE3max, in combination with the candidate DNA repair genes, delivers additional unique hit candidates for iPSC-CMs. This strategy aims to develop optimized editing tools tailored to the unique characteristics of cardiomyocytes, advancing the clinical application of CRISPR nuclease, base, and prime editing therapies for cardiovascular disease.

P0263

Preclinical Data Supporting Efficacy and Safety of EPI-321, an Investigational Epigenetic Editing Product Treating FSHD

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1: Epic Bio

Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common types of adulthood muscular dystrophies without a permanent cure. Aberrant expression of disease causing DUX4 protein leads to apoptosis and progressive degeneration of the skeletal muscle. DUX4 gene is

encoded at the distal 4q35 chromosome from D4Z4 macrosatellite array and in FSHD patients this D4Z4 array is contracted and hypomethylated. EPI-321 is an investigational gene therapy product, a single AAVrh74 vector encoding an ultracompact catalytically inactive Cas molecule fused to a modulator for gene suppression under the muscle specific CK8e promoter and a D4Z4 targeting guide RNA. EPI-321 is designed to address the root cause of hypomethylation and permanently suppress the DUX4 gene expression. In the current studies we evaluated the efficacy of EPI-321 in a humanized xenograft mouse model of FSHD and the safety in immunocompetent C57BL/6 mice. In the efficacy study, a humanized-muscle mouse model transplanted with 3 genetically different patient-derived myoblasts were intravenously dosed at low, mid, and high doses of EPI-321, with an endpoint set at 23 days post-administration. Robust delivery and expression of EPI-321 in skeletal muscle tissue in a dose-dependent manner were confirmed via qPCR and RT-qPCR. Systemic administration significantly reduced DUX4 transcript and protein levels in the xenograft muscle in vivo. Additionally, the expression of DUX4 target genes was markedly reduced, consistent with a lowered rate of cellular apoptosis, demonstrating the therapeutic efficacy of EPI-321 in humanized mouse models. Dose-ranging studies identified the low dose as the minimum efficacious dose, balancing efficacy and potential dose-related side effects. The safety of EPI-321 was evaluated in, male and female C57BL/6 mice aged 8-10 weeks received high-dose EPI-321, followed by in-life examinations throughout the study duration. Tissues for clinical pathology, anatomic pathology, biodistribution, and germline-spatial distribution were collected at endpoints of one- and three-months post-EPI-321 administration. Safety evaluation revealed no abnormalities in in-life examinations or clinical chemistries. Tissue and organ histopathology assessments showed no notable changes at one- and three-months post-administration. Biodistribution analysis confirmed robust and stable transgene delivery across multiple tissues, with specific target tissue transgene expression. Spatial distribution analysis in testes and ovaries confirmed the absence of genetic material in germline cells, ensuring reproductive safety of EPI-321. These preclinical studies provide compelling evidence of the efficacy and safety of EPI-321 as a promising therapeutic candidate for FSHD.

P0264

Natural history of limb girdle muscular dystrophy R9: two-year follow-up of a European cohort

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FKRP-related LGMDR9 (old nomenclature; LGMD2I), is an autosomal recessive limb-girdle muscular dystrophy, characterized by progressive weakness of predominantly proximal limb muscles. LGMDR9 is highly variable in presentation. Individuals can either present a Duchenne muscular dystrophy-like skeletal muscle phenotype with rapid deterioration and loss of ambulation in their teens or present a milder phenotype with onset in late adolescence or adulthood. Individuals with LGMDR9 are prone to early respiratory involvement, with progressive loss of forced vital capacity (FVC). To reinforce published data on the natural history of LGMDR9 and expand the geographic outreach, Genethon conducted an observational study in three European countries (NCT03842878). This study may function as a non-concurrent control for the Atamyo gene therapy trial (NCT05224505). The NHS study enrolled fifty-two ambulant (mean

age 39; range 16-75 years) patients in Denmark, France, and the UK. The mean age at diagnosis was 27.8 years (median 26.5). The mean sitting FVC at baseline was 69.7% of predicted values, the mean walking velocity was 1.61 m/sec (10 MWT) and the mean NSAD score was 30.4. The two-year data was analyzed in 2 subgroups: patients with mild phenotypes at baseline (sitting FVC > 80% and/or NSAD score > 40) versus patients with moderate to severe phenotypes (sitting FVC < 80% and NSAD score < 40). The second group corresponds to the population planned to be enrolled in the gene therapy trial. Evolution of muscular and respiratory outcomes at one year showed a more pronounced drop in mean sitting FVC (-2.7 percent-points), mean NSAD score (-1.6 points), and mean velocity (-13.2%) in the moderate/severe sub-group compared to the milder affected sub-group (-2.5 percent-points, -0.1 points, and +5.5%, respectively). Muscle fat fraction measured by MRI in the lower limbs was the highest in posterior thigh muscles and increased in all lower limb muscles over the follow-up period (the highest increase was in anterior thigh muscles after 2 years). This data supports the eligibility criteria selected for the gene therapy trial and suggests that the primary timepoint for assessing the effects of potentially curative treatment can be at one year provided the population has been carefully selected according to disease severity.

P0265

Trehalose treatment improve microdystrophin gene therapy in a Duchenne muscular dystrophy mouse model

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Duchene muscular dystrophy (DMD) is a muscle degenerative disease that affects mainly boys, which is caused by the loss or drastic reduction in the expression of dystrophin. Gene therapy for the restoration of a functional shortened form of dystrophin (µ-dystrophin) have provided encouraging results in animal models. However, there is still need for improved therapeutic outcomes, notably with complementary treatments to gene therapy. Recently, we performed a non-supervised omics study (plasma microRNAs profiling) of a large DMD cohort and found that cholesterol metabolism is highly dysregulated. In lysosomal storage diseases, lysosomal damage is often linked to cholesterol excess, and interestingly, previous and more recent investigations identified lysosomal perturbations in DMD. Taken together, these observations support the possible link in DMD between cholesterol excess and lysosomal perturbation. In the present work, we observed upregulation of the lysosomal damage biomarker Galectin-3 (Gal-3) in dystrophic muscle of DMD patients' muscle and animal models, and other animal models of muscular dystrophies, thus confirmed Gal-3 as a lysosomal damage biomarker. We then treated mdx mice with Adeno Associated Virus (AAV) expressing µ-dystrophin and found that Gal-3 expression pattern was only partially normalized, supporting an incomplete correction of lysosomal damage by µ-dystrophin gene therapy. Consequently, we treated the mdx mouse by the combination of Trehalose, a safe dietary supplement disaccharide to alleviate lysosomal damage, and AAV µ-dystrophin to restore dystrophin expression, as compared to mice treated by only one of these treatments. We found that correction of lysosomal damage by trehalose exceeded lysosomal correction by µ-dystrophin. Importantly, the best correction of lysosomal damage and muscle function parameters was achieved by the combined treatment. Lysosomal damage may play a key role in the pathology of DMD and other muscular dystrophies and is therefore a potential therapeutic target. Trehalose and gene therapy combined therapy holds the potential for improved therapeutic outcome in muscular dystrophies.

P0266

Using adenine base editors to revert to wild type the LMNA c.745C>T mutation associated to LMNA-related congenital muscular dystrophy

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LMNA-associated congenital muscular dystrophy (L-CMD) is a genetic disorder caused by point mutations in the LMNA gene, for which there is currently no effective treatment. This rare genetic disease manifests as muscle weakness, hypotonia, joint contractures, respiratory insufficiency, spinal rigidity, and cardiac anomalies, often leading to sudden death. Our primary objective is to develop efficient gene therapies for L-CMD. In the past, we have studied the potential of CRISPR 1.0 approaches to destroy the mutant allele and produce and revert the pathologic phenotype. In this study, we explore the potential of base editing technology as a therapeutic approach for this disease. First, we analyzed the specificity of different adenine base editors (ABEs) in combination with different guide RNAs in correcting the mutant allele while minimizing off-target effects. We carried out our studies in different mouse and human cell models carrying one or two copies of the LMNA c.745C>T, p.R249R mutation. Importantly, we investigated the potential generation of the L248P bystander mutation during the base editing process and conducted in-depth analyses of cell clones harboring this variant to assess any resultant phenotypic changes. Our results indicated that while the L248P bystander mutation could be generated, its presence did not result in significant detrimental effects in the studied clones. Our findings provide initial evidence for the potential of base editing as a promising treatment approach for L-CMD, highlighting the importance of addressing possible bystander mutations in developing precise gene therapies.

P0267

Allele-specific epigenome editing in COL6-RD patient-derived primary fibroblasts

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Collagen VI-related dystrophies (COL6-RD) are rare neuromuscular disorders representing a phenotypic spectrum ranging from the severe Ullrich congenital muscular dystrophy to the milder Bethlem muscular dystrophy, with intermediate phenotypes connecting the two extremes. Common symptoms include muscle weakness, impaired ambulation, hypermobility, contractures, and respiratory insufficiency. Most disease-causing mutations are dominant-negative, with one group being point mutations that substitute glycine residues in the triple-helical repeats (Gly-X-Y) essential for the triple-helical domain structure. The assembly of collagen VI involves a multi-step intracellular process from heterotrimeric monomers to dimers and tetramers. Beaded microfilaments are formed after the secretion into the extracellular matrix. In the case of a glycine

substitution, every tetramer incorporating at least one mutated α-chain gets a kink in the triple-helix, leading to intracellular retention.

Since haploinsufficiency does not cause a clinical phenotype, the apparent treatment strategy is to knock down the mutation-carrying allele specifically, enabling the wildtype allele to escape the dominant-negative effect and form more functional tetramers and an extracellular collagen VI network. All published and potential treatment approaches target the mutation directly. However, since COL6-RD is rare and many disease-causing mutations are very rare, a more beneficial approach would be to target the mutated allele specifically, independent of the mutation itself. This project aims to investigate the usage of allele-specific CRISPR-based epigenome editing using common variants for differentiating between the alleles.

First, variants in the regulatory elements of *COL6A2* were identified and phased with the disease-causing mutation for two patient-derived primary fibroblast cell lines harboring different point mutations leading to glycine substitutions. Three shared single-nucleotide variants in the *COL6A2* promoter were chosen to design guide RNAs for the epigenome editor CRISPRoff. After testing different delivery strategies, mRNA transfections were identified as the most efficient and reliable, and transfected cells were further analyzed. The allelic expression was assessed with digital PCR assays that showed that the expression of the mutated allele was significantly reduced for both cell lines. The following methylation analysis through targeted nanopore sequencing revealed that the treatment induced allele-specific methylation of the targeted promotor of *COL6A2*, which was not observed in the control condition. Lastly, the immunofluorescence staining for collagen VI and fibronectin visualized that collagen VI secretion, network formation, and co-localization with fibronectin were restored for successfully treated cells of both patient-derived fibroblasts.

The successful demonstration of phenotypic improvement in cultured patient-derived cells highlights the promise of allele-specific and mutation-independent epigenome editing as a viable treatment strategy for dominant forms of COL6-RD. In the future, the efficiency of the approach has to be increased, and the phenotypic improvement has to be functionally validated in a 3D artificial skeletal muscle model.

P0268

In vivo gene therapy for striated muscle laminopathy

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LMNA mutations induce a group of disorders called laminopathies, most of them affecting striated muscles (SML). All SML present with life-threatening dilated cardiomyopathy, while the age at onset of muscle symptoms is variable, ranging from neonatal period for LMNA-related Congenital Muscular Dystrophy (L-CMD) to an absence of muscle symptoms in isolated dilated cardiomyopathy. L-CMD is the most severe form of striated muscle laminopathy with cardiomyopathy and there is no treatment for L-CMD. We have previously shown the phenotype of KI-*Lmna*^{K32del} mouse model mimicking patient mutation. Mice harbouring this mutation develop an L-CMD phenotype at the homozygous state and isolated cardiomyopathy at the heterozygous state. Taking the advantage of our mouse mode, we have previously reported that the disease pathomechanism involves both lamin haploinsufficiency (decrease lamin A/C expression) and dominant negative effect (expression of toxic mutant lamin A/C). Based on these pathophysiological observations, the present study is focused on the evaluation of a therapeutic

approach that aim both at restoring the normal lamin A/C expression level and reducing the expression of the mutant lamin A/C. We produced AAV2/9 vectors containing human mature lamin A under control of a CMV promoter alone, or in combination with shRNA under a H1 promoter that either specifically targets the p.K32del *Lmna* mRNA, or targets both the WT and mutated mRNA. Systemic administration in new-born mice resulted in a significant increase in maximal survival of homozygous mice. In the heart, lamin A protein level was increased, reaching or overpassing that of wild type. Furthermore, liver level of SREBP1 precursor was normalized. By contrast, in heterozygous mice, despite increased lamin A expression in the heart, none of the treatments led to improvement in terms of survival or cardiac function. The absence of therapeutic benefit at long term is neither due to loss of AAV genome particle nor to a loss of its expression with time. Rather, it is due to side effect in the liver, already reported by others, and to inefficient mouse *Lmna* mRNA knock-down. Based on experiments performed on WT and homozygous mouse myotubes in culture, we hypothesise that the lack of knock-down efficacy is due to inefficient shRNA maturation in mutated cells. Future development will need to consider alternative methods to avoid liver targeting and improve mRNA knock-down efficacy.

P0269

Immunoengineering genetically encoded chimeric PCSK9 vaccine against hypercholesterolemia

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Atherosclerosis, a leading cause of global morbidity, can be, despite its complex etiology, mitigated by lowering blood cholesterol. For many patients application of most common and straightforward therapy, the statins, is unsuitable due to adverse effects (muscle pain, liver damage) or unresponsiveness to therapy. Proprotein convertase subtilisin/kexin type 9 (PCSK9) represents an attractive target for preventive therapy because its inhibition disrupts binding to the LDL-receptor and thus improves LDL-cholesterol clearance. Additionally, deficiency in PCSK9 does not cause any adverse pathology, as demonstrated by therapies with anti-PCSK9 monoclonal antibodies or siRNA. These therapies, however, require multiple administrations (bimonthly for MABs/ biyearly for siRNA), decreasing their wide accessibility. Vaccination, on the other hand, may offer a long-lasting effect. Our primary objective was to develop an innovative vaccine against PCSK9 by designing an “autologous” chimeric protein. By retaining conformation of PCSK9, we aimed to induce the formation of effective antibodies that could neutralize PCSK9-LDLR interaction. Simultaneously, we wanted to prevent systemic autoimmune attacks by cytotoxic T-cells on healthy tissue. First, we transplanted human/murine PCSK9’s solvent-accessible amino acids to a scaffold, consisting of a distant homologue, to generate conformational antibody formation-inducing epitopes. Then, we eliminated mutated computationally predicted $CD8^+$ T-cell epitopes of PCSK9 to avert autoimmune T-cell cytotoxicity. Thus, we obtained a chimeric PCSK9, which, implemented as a DNA plasmid vaccine, generated a humoral immune response in both BALB/c strain as well as in the $ApoE^{-/-}$ transgenic mice, an animal atherosclerosis model. The efficacy of neutralizing antibodies was assessed by the inhibition of PCSK9-LDLR interaction. We analyzed the (lack of) formation and activation of PCSK9-specific cytotoxic T-cells. Vaccination of the atherosclerosis murine model

reduced the PCSK9 and cholesterol serum levels, increased liver LDLR amount, and retained these effects over 24 weeks, resulting in lower atherosclerotic plaque formation in murine aortas. We demonstrated the induction of B-cell tolerance-breaking immune response with chPCSK9, which has a considerably lower propensity for activation of cytotoxic T-cells. In contrast to previously reported peptide vaccine, the chPCSK9 vaccine comprises full protein, thus allowing for the presentation of multiple antibody-inducing epitopes. This work provides the proof-of-concept results for a vaccine against hypercholesterolemia towards the rational vaccine design against endogenous proteins, which could be extended to other therapeutic targets and diseases.

P0270

A novel AAV gene therapy for treatment of MYBPC3 hypertrophic cardiomyopathy

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Mutations in the MYBPC3 gene encoding cardiac myosin-binding protein C (cMyBP-C) can lead to left ventricular (LV) hypertrophy, diastolic dysfunction, cardiomyocyte disarray, heart failure and sudden cardiac death. Most pathogenic mutations of MYBPC3 arise via frameshift, nonsense, or conserved RNA splice site mutations on a single allele and result in protein truncation and reduction in total cMyBP-C protein levels (haploinsufficiency). cMyBP-C is a critical regulator of cross-bridge cycling at its N terminus by altering the interaction between myosin, actin, and-tropomyosin. Although the mechanism linking haploinsufficiency to hypertrophic cardiomyopathy (HCM) development remains unresolved, it is believed that increased Ca^{+} sensitivity and contractility seen in MYBPC3-HCM leads to energy deficiency and compensatory hypertrophy. Restoration of cMyBP-C haploinsufficiency offers a viable therapeutic approach for the treatment of HCM.

We are developing an AAV-based gene therapy using a novel cardiotropic capsid to treat MYBPC3-associated HCM. Unfortunately, effective gene delivery to the heart and safety concerns have been major limitations to clinical translation. Achieving therapeutically relevant levels of cardiomyocyte transduction with IV delivered AAV vectors at doses that are well tolerated has been the field's most pressing challenge. We have identified a novel AAV capsid by a machine learning guided rational design approach that demonstrates significantly improved cardiac transduction at lower doses compared with wild-type AAV9.

While AAV currently represents the most tractable approach to a gene therapy treatment for MYBPC3 HCM, the size constraints of the AAV genome limits the design of therapeutic cargos. Robust transgene expression is often dependent on the inclusion of gene regulatory elements such as post transcriptional regulatory elements and a polyadenylation signal, however the large size of the MYBPC3 gene limits the use of these sequences. As such we have evaluated optimized AAV genomic cassettes utilizing novel promoters and minimized regulatory elements for optimal MYBPC3 expression.

Optimized constructs were tested in our novel cardiotropic AAV capsid with systemic administration in WT mice. We identified one optimized construct that produced >60% expression in the heart and effectively increased cMyBP-C protein levels in the myocardium of

WT mice in a dose-dependent manner. No adverse in-life observations or test article related histopathologic findings were observed in heart, skeletal muscle or liver.

We are currently evaluating our novel capsid and cargo in the context of a MYBPC3-deficient murine model of disease. The MYBPC3-targeted knock-in (KI) mouse model carries the human c.772G4A MYBPC3 transition, which is one of the most frequent HCM mutations and is associated with a poor prognosis. KI mice develop severe LV hypertrophy and systolic dysfunction in the first week of life.

In summary, we have identified a novel cardiotropic capsid and optimized MYBPC3 therapeutic cargo that can achieve broad and therapeutic levels of MYBPC3 in the heart while detargeting off target tissues. Studies are ongoing to confirm therapeutic efficacy in a severe and clinically relevant animal model of genetic HCM.

P0271

Development of a platform gene therapy approach for Congenital Myasthenic Syndromes

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Amplo Biotechnology is developing gene therapies for Congenital Myasthenic Syndrome (CMS) due to mutations in Dok-7 and deficiency of Collagen Q (ColQ). Seeking to increase capital efficacy and timeline to the clinic, Amplo has streamlined the process of testing new adeno-associated virus (AAV) therapeutics by applying a platform approach consisting of similar manufacturing processes and starting reagents (including the same combination of capsid, promoter, and enhancers), analytical and manufacturing methods and only changing the animal model and the transgene delivered. The development of our unified platform approach has proven to be an effective strategy for CMS, leading to the successful pre-clinical development of Amplo's first two gene therapies, AMP-101 (AAV Dok-7) and AMP-201 (AAV ColQ).

P0272

The pre-clinical development of gene augmentation therapy to stabilize the neuromuscular junction in Dok-7 Congenital Myasthenic Syndrome reveals a mechanism of action that has the potential to treat many neuromuscular junction diseases using the same vector

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1: Amplo Biotechnology

Amplo Biotechnology is developing AMP-101 (AAV-Dok-7), the first gene therapy for Dok-7 congenital myasthenic syndrome (Dok-7 CMS). Dok-7 CMS is a rare, disabling, inherited disorder caused by mutations in the Dok-7 protein leading to the failed formation of functional neuromuscular junctions (NMJ). DOK7 CMS symptoms are usually first recognized in infancy with limb-girdle patterns of weakness, ptosis, stridor, vocal cord paralysis, choking spells, and respiratory insufficiency that may require a tracheostomy and respiratory support. Adults present severe diffuse weakness, atrophy, and pronounced scoliosis. By adulthood, The most severe patients show dependency on intermittent respiratory support, tube feeding, and lack of ambulation. Dok-7 CMS's prevalence is estimated to be between 3,000 and 5,000 patients (in the major economies). AMP-101's safety and efficacy have been successfully tested. Mouse toxicology, mouse pharmacology, and non-human primate safety studies. Our work and published evidence suggest that AAV-Dok-7 can benefit the clinical presentation of ALS, Emery-Dreyfuss, SMA, Acetyl Choline Receptor deficiency, and aging; however, until now, it was currently unknown whether Dok-7 activity was conserved in higher species. Here, we report, for the first time, that Dok-7 is not only safe in non-human primates, but it is also active, and it leads to enlargement of the neuromuscular junction. This not only significantly increases the probability of success of AMP-101 for Dok-7 CMS but also reinforces the idea of AMP-101's use in other conditions.

P0273

GJB2 gene therapy-response of two pre-clinical mouse models of the most frequent form of human deafness, DFNB1

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More than 1.5 billion people worldwide live with hearing loss (HL). Sensorineural HL affects one new-born in 700-1000 and approximately one child or young adult in 500 before the age of 20. Approximately 70% of congenital severe to profound deafness cases are hereditary due to monogenic defects, thus potentially treatable by gene therapy (GT).

GJB2-related autosomal recessive non syndromic HL (also referred as DFNB1) is the most common genetic cause of congenital sensorineural HL in many world populations, frequently accounting up to half cases. In addition, it is also responsible for deafness occurring later on, even after 40 years of age. *GJB2* encodes connexin-26 (Cx26) gap-junction channel protein that plays a key role in metabolites and ions homeostasis necessary to cochlear development and sensory hair cell function and survival. Notably, Cx26 contributes to endolymph ionic composition and endocochlear potential, the driving force of the sensory mechanotransduction. Hundreds of pathogenic *GJB2* variants have been reported which outcome spans from mild to profound deafness caused by genomic variants including large deletions, loss-of-function (LOF) and missense variants.

In order to develop GT approaches for deafness caused by *GjB2* defects, we developed several experimental mouse models. Among those that are models for *GJB2* human deafness forms, one *Gjb2*$^{del/del}$ resulting in a biallelic *Gjb2* inactivation mimics the most common form of DFNB1, and the other, *Gjb2*$^{del/Hmut}$, a compound heterozygote, expressing a human missense pathogenic variant (Hmut), corresponds to a frequent human *GJB2* genotype. To circumvent embryonic

mouse lethality caused by *Gjb2* inactivation, conditional knockouts were generated here using the same Cre-recombinant mice which displays a large cochlear spatial expression, i.e. in all Gjb2-positive cells.

Both models showed an elevation of the hearing threshold of 110dB and 90dB on average, in *Gjb2*$^{del/del}$ and *Gjb2*$^{del/Hmut}$ mice, respectively. In both models, inner ear delivery of a GT-*GJB2* recombinant adeno-associated virus (AAV) improved the hearing threshold. Histological analysis showed cochlear structure preservation in both *Gjb2* defective recombinant mice. Especially, treated *Gjb2*$^{del/del}$ mice, GT-*GJB2* fully impeded their dramatic hair cells and supporting cells lost.

Thus, GT-GJB2 improves audition in two preclinical models of human pathogenic GJB2 variants.

P0274

The effect of dexamethasone and tacrolimus on characteristics and immunomodulatory properties of mesenchymal stem cells

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Human mesenchymal stem cells (MSCs) show potential for treating neurodegenerative diseases by producing factors and regenerating in the diseased microenvironment. When injected into the central nervous system, MSCs are known to have immune-privilege properties but can experience a high level of leukocyte infiltration. While immunosuppressants can reduce immune responses to injected MSCs, their effects on stem cell characteristics are poorly understood. Our study aimed to investigate the effects of two immunosuppressant drugs, dexamethasone and tacrolimus, on the characteristics of human mesenchymal stem cells (hMSCs). We also examined whether these drugs could affect the persistence of hMSCs and the immune response when administered into the brain parenchyma of mice for Alzheimer's disease (AD).

MSCs were treated with three different immunosuppressive conditions at various concentrations: dexamethasone, tacrolimus, and a combination of both with varying concentrations. We measured the cell viability of MSCs using the Cell Counting Kit-8 (CCK-8) assay. The tri-lineage differentiation ability of MSCs was evaluated by qualitatively analyzing histological stainings, such as Oil Red O for adipogenic differentiation, Alizarin Red S for osteogenic differentiation, and Safranin O for chondrogenic differentiation. Additionally, we measured the surface antigen expression of MSCs using flow cytometry. hMSCs (2X10^5cells) were injected into the brain tissue of a 5xFAD transgenic AD mouse model and co-administered with a combination of

dexamethasone and tacrolimus. We measured the number of hMSCs left in the mouse brain and analyzed the relative expressions of 64 immune response genes using a screening array.

Our in vitro study revealed that exposing hMSCs to high concentrations of dexamethasone and tacrolimus did not significantly change the characteristics of hMSCs, including morphology, viability, immunophenotype, and differentiation capacity. Additionally, an in vivo study showed that the administration of dexamethasone and tacrolimus did not affect the persistence of hMSCs in AD mouse brains. Moreover, the expressions of genes related to innate immune responses, such as interleukin-1 receptor-associated kinase 1, interferon regulatory factor 3, nucleotide-binding oligomerization domain-containing protein 1, and interferon alpha and beta receptor subunit 1, were significantly reduced in the brain parenchyma of AD mice with the additional administration of dexamethasone and tacrolimus, compared to hMSC alone.

According to the research, immunosuppressants such as dexamethasone and tacrolimus do not affect the quality or longevity of hMSCs. However, they do help to reduce the immune responses that are triggered by hMSCs in the brain. These findings provide evidence that the application of immunosuppressants could be beneficial in controlling fever during stem cell therapy in patients with AD.

P0275

Improved molecular diagnostics of inherited retinal diseases at the transcriptional level using a CRISPR/dCas9-VPR-based approach for ectopic gene activation

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Inherited retinal diseases (IRDs) are a group of genetic disorders that affect the retina, the light-sensing tissue at the back of the eye, leading to progressive vision loss and eventually blindness. Apart from vision, IRDs profoundly affect various aspects of patients' lives, necessitating comprehensive medical, psychological, and social support to manage the disease and its consequences effectively. Since a growing number of therapies are being developed, accurate molecular genetic diagnosis and interpretation are crucial not only for understanding disease mechanisms and developing targeted therapies but also for informed decision-making and patient counseling. IRDs are caused by mutations in more than 270 known genes and this number continues to grow as genetic research advances. This leads not only to the discovery of new genes linked to these conditions but also identifies many genetic variants of unknown pathogenetic significance. To address this diagnostic uncertainty, we employed a novel CRISPR/Cas9-VPR-mediated approach for ectopic IRD gene activation in patient-derived peripheral blood mononuclear cells (PBMCs) that allows for evaluation of the impact of pathogenic variants at the transcriptional level.

To this end, we designed multiple guide RNAs targeting the promoter region of the *ABCA4* and *RPE65* gene which were cloned under the control of the U6 small nuclear RNA promoter. Initial testing of ectopic gene activation was performed in human embryonic kidney cells (HEK293). Our top candidate guide RNAs were subsequently validated in PBMCs from healthy human donors. After isolation of PBMCs using density gradient centrifugation, cells were cultured and nucleofected with all-in-one plasmids containing an endonuclease-deficient Cas9

variant (dCas9) fused to the transcriptional activators VP64, p65 and Rta (VPR) and *RPE65* or *ABCA4* specific guide RNAs. 24 hours after nucleofection, cells were analyzed for gene expression at the mRNA level and specific region amplification via Sanger sequencing and long-read next-generation sequencing (lrNGS, Oxford Nanopore Technologies) was performed.

Both *ABCA4* and *RPE65* genes could be transcriptionally activated in PBMCs of healthy human donors as confirmed by quantitative polymerase-chain-reaction (qPCR). We applied the same protocol to analyze samples (PBMCs) from IRD patients with suspected *ABCA4*- or *RPE65*-associated retinal disease for whom the conventional genetic testing did not provide an unequivocal result. In the patient samples, we have also achieved a good level of transcriptional activation. Short- and long- read sequencing of specific regions of the amplified genes allowed us to analyze the effect of specific mutations at the transcriptional level and confirm the clinical diagnosis.

Taken together, we provide a proof-of-concept for ectopic activation of IRD-linked genes in PBMCs to identify and evaluate mutations from patient samples at the mRNA level. This will help improving the genetic diagnosis of IRD-patients and confirming their clinical diagnosis.

P0276

Genome Editing with AAV-BR1-CRISPR in Postnatal Mouse Brain Endothelial Cells

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Brain endothelial cells (ECs) are an important component of the blood-brain barrier (BBB) and play key roles in restricting entrance of possible toxic components and pathogens into the brain. However, identifying endothelial genes that regulate BBB homeostasis remains a time-consuming process. Although somatic genome editing has emerged as a powerful tool for discovery of essential genes regulating tissue homeostasis, its application in brain ECs is yet to be demonstrated in vivo. Here, we used an adeno-associated virus targeting brain endothelium (AAV-BR1) combined with the CRISPR/Cas9 system (AAV-BR1-CRISPR) to specifically knock out genes of interest in brain ECs of adult mice. We first generated a mouse model expressing Cas9 in ECs (Tie2Cas9). We selected endothelial β-catenin (Ctnnb1) gene, which is essential for maintaining adult BBB integrity, as the target gene. After intravenous injection of AAV-BR1-sgCtnnb1-tdTomato in 4-week-old Tie2Cas9 transgenic mice resulted in mutation of 36.1% of the Ctnnb1 alleles, thereby leading to a dramatic decrease in the level of CTNNB1 in brain ECs. Consequently, Ctnnb1 gene editing in brain ECs resulted in BBB breakdown. Taken together, these results demonstrate that the AAV-BR1-CRISPR system is a useful tool for rapid identification of endothelial genes that regulate BBB integrity in vivo.

P0277

Assessment of MRI-Compatible Infusion Pumps Available for Clinical Trials and Preclinical Studies in Europe Delivering Therapies Intraparenchymally

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When performing a dosing as part of a preclinical study or clinical trial, the delivery system usually comprises: a syringe, an infusion pump, and a delivery device (e.g., cannula, catheter, needle). Many off-the-shelf options are available for routine routes of administration (ROAs) like intravenous infusions, but the delivery parameters are more challenging when conducting direct infusions to the brain tissue or cerebrospinal fluid. The work herein focuses on intraparenchymal delivery needs, as this ROA is associated with the smallest delivery volumes and lowest flow rates (i.e., the most limiting from a technical perspective). Intraparenchymal infusion to the brain is a widely used ROA for gene and cell therapies intended for the treatment of neurological disorders, as it significantly reduces the volume of therapeutic needed and the risk of off-target toxicity, while also ensuring the therapy reaches its intended target.

In addition to the small volume of therapeutic used and the qualities of brain tissue that dictate slow infusion rates, this approach often involves MRI guidance to observe the distribution of therapy within the brain. The infusion pump used therefore needs to be MRI-compatible, with the ability to deliver at a microliter level with high resolution. Most infusion pumps cleared for clinical use in Europe are not suitable for MRI-guided intraparenchymal infusions, due to insufficient accuracy, inability to deliver at low flow rates, lack of MRI compatibility, or prohibitively high hold up / dead volumes required.

The work herein presents the validation of commercially-available pumps to confirm the accuracy and reliability of gene therapy delivery at the intended flow rates and volumes used for intraparenchymal infusions. To leverage a clinically-relevant delivery system, three pumps with regulatory clearance were tested with a SmartFlow Cannula and a various syringes to infuse a diluent comparable in viscosity and makeup to that of an AAV-based gene therapy. The three pumps used were the B. Braun Perfusor, the Medfusion 3500, and the MedCaptain HP-30 Neo, and the accuracy of the system was tested by infusing into an agar gel that mimics the backpressure of brain tissue. The results presented herein establish that the pumps tested can achieve acceptable accuracy for the delivery volumes and flow rates used in gene therapy clinical trials. Two of the pumps (B. Braun and MedCaptain) are CE Marked, thereby providing trial sponsors in Europe with two viable commercially-available options for their intraparenchymal delivery system.

P0278

Endogenous CRISPRa-mediated optogenetics restores behavioral vision in a blind mouse model

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Photoreceptor degeneration due to inherited and multifactorial pathologies ultimately leads to blindness. For example, the highly heterogeneous retinitis pigmentosa (RP) is characterized by progressive death of photoreceptors. However, inner retinal cells remain anatomically and functionally intact for an extended period of time, rendering inner retinal cells potential targets in late stages of photoreceptor degeneration. Even though the handicap of affected patients is tremendous, there currently exists no cure. CRISPR-Cas–mediated transactivation of genes provides an opportunity to express endogenous opsins within preserved inner retinal cells, effectively transforming them into replacement light sensors.

We employed split-intein dCas9-VPR with *in vitro* evaluated single-guide RNAs (sgRNAs), delivered by AAVs through intravitreal injections into degeneration 1 (rd1) mice to transactivate the middle-wave cone opsin gene (*Opn1mw*) in retinal ON-bipolar cells (OBCs). Our results show efficient transactivation *in vivo* and restoration of behavioral vision in treated blind *rd1* mice, demonstrating the potential of CRISPRa to transactivate gene expression in vivo sufficiently for functional restoration. As opposed to a supplementation gene therapy, the CRISPRa approach profits from engodenous regulation of protein expression, reducing the risk of cytotoxicity.

P0279

Mesenchymal Stromal Cell potential to restore autophagy and mitochondria health in Machado-Joseph disease

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Machado-Joseph disease (MJD), also known as Spinocerebellar ataxia type 3 (SCA3), is a neurodegenerative disorder caused by an overexpansion of the CAG repeat in the *MJD1/ATXN3* gene. This results in an abnormally long polyglutamine tract in ataxin-3 protein, leading to neurodegeneration. Currently, there is no disease-modifying treatment available. Previously, we demonstrated that Mesenchymal Stromal Cell (MSC) treatment ameliorates the phenotype of MJD transgenic mice, though the mechanisms behind these neuroprotective effects remain unclear.

In this study, we aimed to elucidate MSC's potential to modulate autophagy and mitochondrial health in both *in vitro* and *in vivo* MJD models.

The *in vitro* studies involved direct or indirect co-cultures of neuronal cells expressing a truncated mutant ataxin-3 protein with MSCs. Our results revealed that MSC treatment improved the severely impaired autophagic flux in a neuroblastoma cell line (N2a) expressing mutant ATXN3

(N2a Mut), evidenced by a reduction in the number of degradative autophagic vesicles, which was reverted by MSC administration. Additionally, we found evidence of MSC transfer of mitochondria directly to N2a Mut cells (co-culture experiments). Finally, live imaging confirmed that MSC-conditioned medium enhanced mitochondrial dynamics in hippocampal neurons expressing mutant ATXN3. Furthermore, *in vivo*, 8-month-old MJD transgenic mice exhibited dysregulation in mitophagy and mitochondrial dynamics in their cerebella. Repeated intravenous administration of MSC (two to three treatments) partially reversed these alterations. Specifically, the abnormal levels of the mitophagy markers phosphorylated Parkin and PINK1 were normalized by MSC treatment, indicating improvements in mitophagy. Furthermore, the mitochondrial proteins MFN2 and DRP-1 were also re-established by MSC administration.

In conclusion, our findings provide compelling evidence of dysfunction in autophagosome maturation and mitochondrial turnover in MJD. MSC-based therapy shows potential in reversing these abnormalities, offering a promising therapeutic avenue for MJD.

P0280

Effective *SOD1* targeting with vMiX™, an innovative AAV-based RNA interference platform

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Amyotrophic lateral sclerosis (ALS) is a severe and fatal neurodegenerative disease characterised by the progressive loss of upper and lower motor neurons leading to muscle atrophy, paralysis, and respiratory failure. Around 90% of cases are considered sporadic (sALS) but 10% have an affected relative and are considered to have familial disease (fALS). ALS is a genetically heterogeneous disease with pathogenic mutations reported in 17 genes to date in familial and sporadic cases. The first gene to be linked to ALS was *SOD1*, encoding Cu/Zn superoxide dismutase 1 (SOD1), an antioxidant enzyme predominantly localised to the cytoplasm. While more than 180 *SOD1* mutations described to date account for 12-20% of fALS and 1-3% of sALS cases, specific mutations can influence the age at onset and rate of disease progression. All mutations studied lead to SOD1 protein misfolding and aggregation, causing a toxic gain-of-function to multiple pathways essential to motor neuron survival. The recent positive EMA CHMP opinion and accelerated approval from the US FDA for Qalsody (Tofersen) were based on reductions in the levels of SOD1 in cerebrospinal fluid (CSF), reduction in plasma levels of neurofilament light chain (NfL), and a numerically favourable effect on ALSFRS-R clinical scale. This landmark approval confirms the concept of gene silencing as a rational therapeutic strategy for SOD1-ALS, and is supportive of a potential one-time only administration of gene silencing vectors as an alternative approach with potential to reduce treatment burden in the longer term.

We have developed a novel vector platform harnessing microRNA (miRNA) interference to silence gene expression (vMiX™). The design of this platform was initially optimised to improve miRNA processing to produce an efficient target knockdown. To evaluate the platform *in vitro*, 16 miRNA guides targeting across the coding sequence of *SOD1* were designed and cloned. Initially, we employed a luciferase assay to assess the efficacy of *SOD1* knockdown by these guides through co-transfection in HEK293 cells. The eight guides which most effectively reduced luminescence were selected for further *in vitro* analysis, all of which successfully reduced endogenous *SOD1* expression in HEK293 cells by up to 90% compared to a non-targeting miRNA.

We subsequently produced AAV9 vector of these candidates, which successfully reduced *SOD1* expression in HEK293, HeLa and SH-SY5Y cells following transduction.

Consequently, an *in vivo* study was performed using the B6SJL-Tg(*SOD1*^{G93A}) fALS model by bilateral intracerebroventricular (ICV) vector administration in neonatal mice. After six weeks, analysis of cortical and spinal cord samples demonstrated an efficient knockdown of *SOD1* mRNA across a broad range of vector genome/cell quantifications. Additionally, small RNA sequencing and subsequent miRNA processing analysis showed favourable guide:passenger ratios for these vectors both *in vitro* and *in vivo*.

We have developed and demonstrated that vMiX™, a novel RNA interference platform with the capacity to efficiently silence a gene associated with both familial and sporadic ALS, has potential as a future therapeutic strategy. Furthermore, this platform shows broad adaptability for an AAV-based RNA interference approach to target a range of diseases.

P0281

Gene Therapy For Spinocerebellar Ataxia 7: Restoring Cholesterol Metabolism

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Brain cholesterol is almost exclusively synthesized *in situ* and defects in this metabolism may contribute to neurodegenerative disease such as Alzheimer's (AD) and Huntington's (HD). Previous data in rodent and human patients have demonstrated the impairment in AD and HD of CYP46A1, the brain-specific rate-limiting enzyme in the degradation of cholesterol. In AD and HD mouse models restoring the CYP46A1 enzyme is neuroprotective. Like HD, Spinocerebellar ataxias (SCAs) are caused by the expansion of a translated CAG (polyQ) in the respective proteins. SCA7 is caused by pathological repeat (34 to >200 polyQ) in the ATXN7 protein and characterized by cerebellar ataxia with retinal degeneration.

Our work aims to investigate the role of brain cholesterol metabolism dysfunction in the ATXN7 140Q Ki mouse model and evaluate if overexpressing CYP46A1 enzyme thanks to an AAVPHP.eB using intravenous delivery could cure or alleviate SCA7 pathology.

To assess the gene therapy potential on the SCA7 disease progression, we assessed monthly several parameters characterizing the SCA7 phenotype including body weight, locomotion, motor skills and vision. After 9 months, at the end of the behavioural analysis, we performed electrophysiological analysis of neuromuscular function and vision prior to brain collection for further histology, biochemistry and lipidomic analysis. Results show that overexpression of CYP46A1 leads to increasing the level of intermediate compounds of the brain cholesterol metabolism while improving locomotion, motor skills, and neuromuscular reflexes.

P0282

AAV-based ERCC6 supplementation as a potential gene therapy for Cockayne Syndrome

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Cockayne syndrome B (CSB) is a rare, multisystem disorder, with autosomal recessive inheritance. This progeroid syndrome arises when the disease-associated gene, excision repair cross-complementing protein group 6 (*ERCC6*), is mutated. The main clinical features of CSB include severe growth deficits, neuropathological abnormalities, sensorial impairment, musculoskeletal defects, cutaneous photosensitivity, and a distinct facial appearance with deep sunken eyes. ERCC6 plays major roles in chromatin remodeling and DNA damage repair mechanisms. Thus, Cockayne syndrome is admitted resulting from the unchecked accumulation of DNA damage, which culminates in premature aging and frequently juvenile death. Therefore, there is an unmet need for the development of therapeutic strategies to halt disease progression or permanently treat the disease. The main goal of this work is to develop a disease-modifying gene augmentation therapy by gene delivery of an AAV vector encoding a functional copy *ERCC6*. However, the *ERCC6* cDNA and the plasmid regulatory elements combined size is beyond the AAV optimal packaging size. To circumvent this, we sought to clone different promoters and polyA sequences into a pAAV backbone containing *ERCC6* cDNA. Promoters with different sizes and expression strengths, as well as different polyA sequences were employed to generate through molecular cloning in a total of 3 distinct ERCC6-expression cassettes (Termed Cure1 to Cure3). The ability of the 3 employed promoters to drive *ERCC6* cDNA expression was first assessed *in vitro*. HEK293T ERCC6 KO were transfected with the different ERCC6-encoding plasmids and cell lysates posteriorly collected for western blot analysis. Western blot analysis of Cure1 transfected cells revealed increased ERCC6 protein levels, compared to non-transfected cells. However, Cure2 and Cure3 transfected cells did not show an increase in ERCC6 protein levels. The brain is specially affected in CSB, as such to harness neuronal tropism and the ability to cross the brain-blood-barrier our strategies were packaged into recombinant AAV9. To evaluate the strategies viability and their ability to express ERCC6 *in vivo*, the viral particles were delivered to the striatum of *ERCC6*$^{m/m}$ mice through unilateral stereotaxic surgery ($9x10^9$ vg/per animal). Surgeries were performed in adult animals, and the brains collected 4 weeks post injection. Whole brains were collected for immunohistochemistry, while striatum punches were collected for western blot analysis. Immunohistochemistry analysis showed that Cure1 injected hemisphere displayed robust ERCC6 expression in comparison to the non-injected hemisphere. In contrast, we failed to detect ERCC6 expression in Cure2 and Cure3 injected mice. Accordingly, western blot analysis of Cure1 injected striatum punches displayed increased ERCC6 protein levels relative to non-injected control. These finding indicate that despite surpassing the ideal AAV packaging size, Cure1 is able of efficiently driving ERCC6 expression *in vivo*. Altogether, the ERCC6-encoding strategy, Cure1, has the potential to be employed as a gene augmentation therapeutical strategy in pre-clinical *in vivo* studies.

P0283

Evaluation of mutant Huntingtin knockdown after intra-striatal administration of AAV-miHTT constructs in the YAC128 mouse model of Huntington's Disease

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Huntington's disease (HD) is caused by a mutation in the *Huntingtin* gene (HTT) that abnormally increases the number of CAG nucleotide repeats. Consequently, the translated protein's exon 1 contains an expanded polyglutamine (polyQ) tract, resulting in a toxic gain of function which makes the protein prone to misfolding and aggregation, affecting its normal cellular function and ultimately leading to neuronal death.

Different studies have shown that lowering mutant HTT in the brain of HD rodent models could improve neuropathological and behavioural abnormalities. However low levels of wild-type HTT, is responsible for a loss of function of the protein, which contributes to the pathogenesis. Thus, targeting and reducing the levels of mutant HTT, while minimizing the effect on the reduction of wild-type HTT, has been viewed as a promising therapeutic strategy.

This study aimed to assess the silencing effectiveness of a selected miRNA sequence (miHTT-4) by quantifying the levels of expanded mutant human Huntingtin (HTT) and total HTT in the striata of YAC128 mice. The silencing efficiency of miHTT-4 was compared to a control miRNA sequence (miHTT-12) previously shown to be able to reduce HTT levels in HD mice. Two distinct titers were administered for each of the miHTT sequences (Low titer: 1.6E9 vg/µL versus High titer: 6.0E9 vg/µL). The control YAC128 group was administered vehicle.

The analysis of soluble mutant HTT in YAC128, which received either miHTT-4 or miHTT-12, demonstrated that the levels of silencing were similar for both miHTT sequences. At the lowest titer, the levels of miHTT were reduced by 27% and 34% for miHTT-4 and miHTT-12 respectively, in comparison to the control group. YAC128 mice that received a high titer of either miHTT-4 or miHTT-12 showed a reduction of 39% and 47% respectively in the levels of soluble mHTT compared to vehicle treated mice.

When YAC128 mice were given either miHTT-4 or miHTT-12 at the lowest titer, the total HTT levels were shown to decrease by 13% and 17% respectively. YAC128 mice getting the highest titer of miHTT-4 showed a decrease of 19% in total HTT levels, whereas the lowering levels were higher in mice miHTT-12 at the high titer (34%).

Overall, the data suggests that the efficacy in lowering mHTT using the selected miRNA sequence (miHTT-4) is similar to the one obtained with of the control sequence miHTT-12 Nevertheless, the decrease in total HTT levels appears to be restricted for miHTT-4, suggesting a comparatively smaller reduction in wild-type HTT levels. However, this observation will require additional investigation.

P0284

Broad CNS Biodistribution of AAV9-based Gene Therapies Delivered by Intrathecal Lumbar Puncture in Non-Human Primates

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Direct intraparenchymal or intra-cerebrospinal fluid (intra-CSF) dosing has been explored for delivery of recombinant adeno-associated virus (rAAV9) vectors into the CNS, bypassing the blood-brain barrier. While intra-cisterna magna (ICM) injection and other CNS-directed routes have been considered, intrathecal (IT) delivery by lumbar puncture is preferred as a minimally invasive approach for clinical use. To evaluate vector delivery, we have retrospectively quantified CNS biodistribution of rAAV9 vectors in >60 non-human primates (cynomolgus macaques; NHPs) across 5 studies, allowing comparisons between routes of administration.

During preclinical characterization of investigational gene therapies, biodistribution in NHPs of TSHA-101, TSHA-102, TSHA-105 and TSHA-120 was evaluated after administration by the IT (all products) or ICM (TSHA-102 only) routes. TSHA-120 was administered via IT at the lowest doses (0.3 and 1.16 x 10^{13} vector genomes (vg) per animal (corresponding to 0.26 and 1.0 x 10^{14} vg human-equivalent doses (HED), respectively). For the other vectors, doses of 5.77 x 10^{13} (HED 5 x10^{14}) vg were used. A higher dose, 2.31 x 10^{14} (HED 2.0 x 10^{15}) vg was also explored following IT (TSHA-102 and TSHA-105) and ICM (TSHA-102) administration. CNS distribution was assessed following scheduled necropsy at 30 (TSHA-101), 90, and 180 days (TSHA-102 and TSHA-105) post-injection, using qPCR to quantify rAAV9 DNA. Protocols for analysis differed: whereas TSHA-120 was examined in 15 sections along a rostro-caudal axis, TSHA-102 (IT dosing) was examined in two brain regions; the other biodistribution studies quantified rAAV9 at 6 brain sites (frontal, parietal, and occipital lobes; temporal and prefrontal cortex; and cerebellum) and 3 spinal cord (SC) sites (cervical, thoracic, and lumbar SC).

Following IT delivery, TSHA-101 and TSHA-105 vector DNA was detected in all brain regions analyzed (mean 10^{5} to 10^{6} vg/µg). TSHA-102 vector DNA levels at 90 and 180 days were comparable to those of TSHA-101 and TSHA-105 (~1.5 – 3.0 x 10^{5} vg/µg at 5.77 x 10^{13} dose). At Day 90, TSHA-120 at both doses was detected at generally uniform levels in all serial sections. At each necropsy time point, detection of TSHA-102 and TSHA-105 vector DNA in brain tissues varied with administered IT dose, such that a 4.5-fold dose increase was associated with a 1.5- to 2.5-fold vector copy number increase in frontal and parietal lobe tissues. Similarly, following ICM administration of TSHA-102, vector DNA was detected in all 9 brain/SC regions analyzed. Average vector DNA copy number was ~5 x10^{5} vg/µg at 2.31 x 10^{14} vector dose across all brain tissues. For each rAAV9 examined, vector DNA was detected in SC as well as brain.

Overall, biodistribution analysis in Taysha's NHP studies showed that both the IT and ICM routes led to comparable widespread AAV9 distribution throughout the CNS, achieving brain levels of ~3 – 5 x 10^{5} vg/µg at comparable doses and timing. These findings support the use of lumbar IT administration as an effective, procedurally simple approach for rAAV9 dosing of the CNS.

P0285

Gene augmentation therapy to preserve hearing in a murine model of Norrie Disease

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Norrie Disease is a rare X-linked recessive disorder caused by mutations in the Norrie disease pseudoglioma (*NDP*) gene. The symptoms of the disease consist of congenital blindness followed by sensorineural hearing loss typically occurring from the teenage years onwards. This dual sensory loss is debilitating and severely impacts patients' quality of life. No curative or preventative treatments are currently available for Norrie disease, however the later onset of the deafness presents a potential therapeutic window to preserve hearing.

Using a mouse model of Norrie Disease (*Ndp*-KO) we have previously investigated the onset of histological and physiological changes constituting the Norrie disease auditory phenotype [PMID 35132964]. Norrie disease mice display abnormal morphology of the cochlear microvasculature including spiral ligament and stria vascularis capillaries and impaired function of the vascular barrier in the cochlea. Over time this is followed by the loss of sensory hair cells in the cochlea and consequently irreversible hearing loss. In recent proof-of-concept studies [PMID 37642150] we showed that gene augmentation therapy for Norrie disease delivered systemically using the AAV9 capsid was able to prevent progression of the hearing loss in treated mice when administered across a range of timepoints. Treatment at postnatal day (P) 2 achieved complete preservation of hearing while partial preservation was achieved after treatment at P30, mirroring the higher level of transduction achieved at P2 than P30.

In this study, we evaluated the efficacy of *NDP* gene therapy using the modified AAV-S capsid, which is reported to have an efficient transduction profile for the mouse ear [PMID 33869656]. We tested whether use of the AAV-S capsid at P30 completely preserved hearing. *Ndp*-KO mice were injected at P30 with the AAV-S viral particles containing gene therapy construct EGFP-P2A-hNDP. Evaluation of these mice was undertaken involving: auditory brainstem response testing to assess the hearing loss phenotype; histology to examine hair cell preservation; and qPCR analysis of disease biomarker genes expression (*Cldn5*, *Plvap*, *Abcb1a*, *Sox17*) associated with the cochlear microvasculature phenotype. We show that AAV-S transduction achieved *NDP* expression in the *Ndp-KO* inner ear. The gene therapy partially restored dysregulated cochlear gene expression and ameliorated hearing loss. Hearing levels were intermediate between the wildtype and untreated *Ndp-KO* mice by 2 months.

P30 corresponds to early Norrie disease stage in young patients, therefore testing treatments at this age is clinically relevant and important for future clinical trials. This study supports the proposal that early treatment of the Norrie disease phenotype by an AAV mediated gene therapy could reduce the progression of the cochlear pathology and improve the level of auditory function in patients.

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P0286

Developing a Progranulin AAV Gene Therapy for Amyotrophic Lateral Sclerosis

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Dominant mutations in progranulin (PGRN) result in haploinsufficiency, with most mutations leading to a 50% reduction in PGRN levels, causing frontotemporal dementia (FTD). Recently, mutations in PGRN have also been identified as a risk factor for amyotrophic lateral sclerosis (ALS), placing PGRN within the ALS-FTD clinical spectrum. Moreover, the presence of ubiquitinated TDP-43 deposits is the neuropathological marker frequently associated with these PGRN mutations, demonstrating a link between PGRN dysfunction and aberrant TDP-43 accumulation. The PGRN is processed into 7.5 granulin (GRN) small ~6kDa cysteine-rich peptides, which are separated through proteolytic cleavage. These granulin peptides can function independently and have distinct biological activities. In this study, we engineered individual GRNs that are combined uniquely to generate GRN-X transgenes. We then tested whether the GRN-X could change the cytoplasmic localisation and insolubility of the TDP-43 M337V in various cell lines. We discovered that GRN-X formed granules of various sizes and were colocalised with subcellular lysosomal markers. We are currently selecting the best candidate for the TDP-43 M337V and developing the adeno-associated virus (AAV) that delivers the engineered GRN-X to TDP-43 iPSCs and transgenic mice. Furthermore, we have developed an assay to measure the immune response using Lipopolysaccharide (LPS) in the microglial cell line, BV2, to assess the anti-inflammatory effect of the engineered GRN-X. In conclusion, we are conducting studies to identify which GRN-X transgene can reduce the aberrant accumulation of TDP-43 protein for ALS AAV gene therapy.

P0288

GBA1-linked Parkinson’s Disease AAV gene therapy

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Parkinson’s disease (PD) is a chronic, progressive, neurodegenerative disorder characterised by α-synuclein accumulation, Lewy Body formation and loss of dopaminergic neurones within the substantia nigra pars compacta (SNpc) which controls movement and coordination. Non-motor symptoms include cognitive decline, sleep disturbance and psychiatric symptoms. Mutations in *GBA1* are linked to Gaucher disease (GD), one of the most common lysosomal storage diseases. In this population, approximately 9.1% of patients are likely to develop PD before the age of 80, compared to 3-4% for the wider population[1]. Heterozygous *GBA1* GD mutation carriers and a number of non-Gaucher PD associated *GBA1* mutations share a similar level of risk of developing PD. A proportion (5-15%) of people with PD carry mutations in *GBA1* which encodes the lysosomal enzyme glucocerebrosidase (GCase), resulting in dysfunctional enzyme and lysosomes[1].

AAV-mediated gene therapy providing sustained endogenous levels of fully functional GCase may reduce the early and accelerated rate of disease progression observed for *GBA1*-linked PD. Our GCase variant 85 ($GCase_{var85}$), with two amino acid substitutions to the mature human GCase ($GCase_{WT}$), has been established as a more stable enzyme[2] with clinical benefit in GD and offers the potential for effective delivery to and improved distribution across the brain in comparison to $GCase_{WT}$. *In vitro* transduction of brain derived cell lines, including SH-SY5Y (neuroblastoma), demonstrated up to 10-fold or more GCase activity in cell supernatants with AAV9-$GCase_{var85}$ compared to AAV9-$GCase_{WT}$ across all cell types, reflecting the improved stability.

Expression and distribution were evaluated in C57BL/6J male mice of 9 weeks of age, injected unilaterally into the right-hand caudate putamen with vehicle or AAV9-GFP, AAV9-$GCase_{WT}$ or AAV9-$GCase_{var85}$ at a dose of 1.3×10^{10} vg per mouse and analysed four weeks post dose. Whole brains were coronally cryosectioned at the striatal site of injection and the substantia nigra and sections were immunofluorescently labelled for GCase or GFP. The AAV9-GFP reporter demonstrated effective retrograde transport from the caudate putamen, with GFP widely distributed in axons of the substantia nigra. Qualitative immunofluorescent staining for GCase gave a more intense signal in numerous somata and neuropil of the injected right hemisphere and signal in the left hemisphere for AAV9-$GCase_{var85}$ compared to AAV9-$GCase_{WT}$, with a clear GCase signal in tyrosine hydrolase-positive somata in the substantia nigra pars compacta of animals from both groups. Biochemical analysis of GCase activity extracted from the right hemisphere striatum or substantia nigra of treated animals demonstrated approximately 10-fold higher activity for AAV9-$GCase_{var85}$ compared to AAV9-$GCase_{WT}$ in agreement with *in vitro* observations.

In conclusion, direct brain injection of AAV9 constructs to the caudate putamen is effectively distributed to the target cells of the substantia nigra. The more stable $GCase_{var85}$ results in a 10-fold higher GCase exposure with broader distribution across the murine brain.

P0289

Efficacy evaluation of Wharton's Jelly-derived Mesenchymal Stem Cells with improved therapeutic effects *in vitro* model of Alzheimer's Disease

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Alzheimer's disease (AD) is the most common neurodegenerative disease and is related to multiple pathogenesis. As shown in numerous studies, mesenchymal stem cells (MSCs) that secrete bioactive molecules with diverse therapeutic effects demonstrate enhanced efficacy in treatment with fewer adverse effects compared to therapeutic agents that target a singular pathway. AD is characterized by deposition of amyloid beta (Aβ) aggregates and hyperphosphorylated tau protein tangles in the brain. The clearance of Aβ, which is the initiator and trigger of disease development, is known to be an appropriate potential marker for evaluating

potential therapeutic efficacy. In this study, we aimed to confirm the therapeutic effect of MSCs with improved efficacy by measuring the level of Aβ secreted by cells in a genetically engineered AD H4 cell line model (H4SW cell), rather than using synthesized Aβ protein. Iron oxide nanoparticles (IONP), which are FDA-approved nanomedicines, are substances widely used as treatment-related substances. These nanoparticles are non-toxic to the body, possess decomposition properties, and have recently been utilized safely to improve the therapeutic efficacy of cells. The results of this study demonstrated that co-culturing MSCs with IONP (IONP-MSCs) with the H4SW cell line resulted in a decrease in Aβ levels and an increase in neprilysin (NEP) levels, the enzyme involved in Aβ degradation, compared to naïve MSCs, as measured by ELISA. Furthermore, analysis of inflammation-related markers, which are major factors in AD, revealed that the levels of representative inflammatory cytokines TNF-α and IL-1β decreased following the co-culture of IONP-MSCs with H4SW cells. Therefore, the findings of this study are expected to demonstrate the therapeutic potential of Wharton's jelly-derived MSCs with improved efficacy through IONP treatment in an AD *in vitro* model.

P0290

Advancements in preclinical gene therapy

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The field of gene therapy is undergoing rapid advancement and thus holding immense promise to revolutionize treatments across a spectrum of genetic disorders. As a leading Contract Research Organization (CRO) specialized in preclinical research, our mission is to facilitate the translation of groundbreaking scientific discoveries into tangible preclinical applications.

Our capabilities encompass a comprehensive toolbox enabling the optimization of embracing both innovative viral and non-viral delivery systems meticulously crafted to optimize transgene expression and tissue specificity. Leveraging state-of-the-art *in vivo* and *in vitro* models, we strive to identify and validate therapeutic targets with greater precision.

Distinguished by our proficiency in rodent surgical techniques, we offer accurate administration of gene therapy agents, ranging from parenteral to stereotactic injections with a dual focus on maximizing efficacy while minimizing invasiveness. Moreover, we even offer the possibility to administer gene therapy agents to larger animals such as rabbits and soon also to minipigs. Despite prevailing challenges such as those pertaining to delivery and durability, our commitment to transparency, clarity, and adaptability remains unwavering throughout all phases of gene therapy studies.

At the core of our proficiency lies our extensive expertise with adeno-associated viral (AAV) vectors, both in vivo and in vitro, augmented by a highly skilled team that is specialized in histological and biochemical analyses to support comprehensive ex vivo evaluations for gene therapy studies.

Moreover, quantitative Polymerase Chain Reaction (qPCR) can be performed to quantify vector presence and distribution within target tissues. Soon, we will introduce Fluorescence *In Situ* Hybridization (FISH) for the detection of AAV vectors provides spatial information on vector localization, offering valuable insights into post-administration vector behavior. Additionally, the evaluation of target protein expression can be explored through biochemical and histological approaches, allowing for the assessment of therapeutic efficacy and protein functionality at

molecular and tissue level. Finally, we are currently establishing Magnetic-Activated Cell Sorting (MACS) for the isolation of specific cell subpopulations such as microglia. These advancements represent significant steps to enhance the precision and effectiveness of gene therapy, paving the way for an improved therapeutic outcome.

Central to our endeavors is the paramount consideration of safety. We present robust data derived from thoroughly conducted preclinical in vivo studies which underscores the importance of accuracy and reliability for all methods applied. In general, years of experience with clients all around the world ensure that our preclinical models consistently surpass industry standards, thereby expediting regulatory approval processes and streamlining the journey to market for novel therapies.

P0291

Assessing the efficacy of lentiviral vectors encoding chimeric human GALC enzyme to optimize hematopoietic stem cell gene therapy for globoid cell leukodystrophy

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Globoid cell leukodystrophy (GLD) is a fatal lysosomal storage disorder caused by a deficiency of the β-galactosylceramidase (GALC) enzyme, resulting in severe central and peripheral nervous system dysfunction. The main challenge in treating GLD is achieving sufficient GALC activity in affected tissues, which limits the effectiveness of gene therapy (GT) strategies. To address this issue, we engineered GALC enzymes to enhance their secretion and ability to cross the blood-brain barrier, potentially increasing GALC availability and improving GT outcomes. We designed and evaluated the safety and efficacy of lentiviral (LV) constructs encoding engineered chimeric human GALC enzyme in CD34+ hematopoietic stem and progenitor cells (HSPCs) and in neural cells generated from GLD patient-induced pluripotent stem cells (hiPSCs). These models represent the closest human in vitro approximations of effector and target cells in HSPC-GT approaches. Safe overexpression of transgenic mRNA in LV-transduced human cells correlated with supranormal GALC activity. HSPCs and hiPSC-derived neural progeny secreted the chimeric GALC enzymes more efficiently than their unmodified counterparts. Notably, GLD patient-derived neurons/glial cells recaptured the chimeric GALC enzyme proficiently, achieving GALC levels close to physiological values upon cross-correction. When LV-transduced CD34+ HSPCs were xenotransplanted into immunodeficient NSG mice, the human-derived cells successfully engrafted, constituting up to 20% of the peripheral blood cells after eight weeks. Importantly, we observed increased GALC activity in the blood serum of mice that received CD34+ HSPCs expressing the chimeric human GALC enzyme compared to those treated with the unmodified version. This indicates enhanced secretion and circulation of the chimeric GALC enzyme in the bloodstream. These findings encourage further exploration into the safety and advantage of chimeric GALC enzyme in HSPC-GT approaches in GLD mouse models. Enhanced GALC availability could reduce the need for high levels of GALC overexpression in effector cells, simplifying clinical protocol development and improving safety profile.

P0292

Targeting multiple pathogenic pathways in Huntington's disease using linQURE®: a feasibility study in rodent disease models

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Huntington's disease (HD) is an autosomal dominant disorder caused by an expanded CAG repeat in the huntingtin (*HTT*) gene, with full penetrance above 39 CAG repeats. The unstable CAG repeat expansion leads to a toxic gain of function of *HTT* mRNA and protein species, causing cellular dysfunction and neurodegeneration. There are currently no disease modifying therapies for HD. Many therapies under development are designed to lower (mutant) HTT levels with the goal of slowing neurodegeneration. Recent studies indicate that, besides mutant HTT, somatic repeat expansion through aberrant mismatch repair may be pivotal disease contributors, with MutS homolog 3 (MSH3) as a promising therapeutic target. Here, we have explored the feasibility of simultaneous dual or triple targeting of (mutant) *HTT* and *MSH3*, using our linQURE® AAV platform, as a next-generation therapeutic approach for HD. Therapeutic microRNAs targeting *HTT1a* or *MSH3* were designed and screened *in vitro*. Lead candidates were selected based on potency and specificity and evaluated in relevant HD mouse models. We tested individual *HTT1a* targeting in YAC128 mice, showing potent reduction of mutant HTT and HTT1a proteins. Individual *Msh3* targeting was tested in *Hdh*Q111 mice, showing potent reduction of *Msh3* and somatic repeat instability. We then proceeded to testing multi-target approaches using linQURE®, first in WT mice, showing good expression of both or all three therapeutic microRNAs from the same vector. Finally, dual *Msh3* and *HTT* targeting was tested in R6/1 mice, and dual and triple *Msh3*, *HTT* and *HTT1a* targeting was tested in BAC-CAG mice, in all cases leading to efficient lowering of the therapeutic targets. The present study demonstrates the feasibility of targeting multiple therapeutic candidates from a single vector using linQURE®, and holds promise as potential next-generation AAV gene therapy for HD.

P0293

HSC-derived CAR-Treg gene therapy: a novel approach for the treatment of multiple sclerosis

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Autoimmune diseases such as multiple sclerosis (MS) are increasing in prevalence, yet currently only non-specific anti-inflammatory drugs are used to manage patient symptoms, and no long-term durable or curative therapies are available for this chronic debilitating disease. Adoptive transfer of regulatory T cells (Tregs) expressing chimeric antigen receptors (CARs) is a novel strategy for the treatment of MS. By introducing CARs that recognise autoantigens into Tregs,

their immunosuppressive function can be both targeted and enhanced allowing immune homeostasis to be restored. While this approach has shown promise, it is limited by the potential stability of CAR-Tregs post transfer and their long-term efficacy. We are developing a novel CAR-Treg therapy for the treatment of MS, harnessing our autologous haematopoietic stem cell gene therapy (HSC-GT) platform.

Using lentiviral vectors to transduce HSCs, CAR-Tregs can develop naturally *in vivo*, following transplantation of HSCs into conditioned recipients. This is achieved by the introduction of engineered promoter elements that control and restrict CAR expression to the Treg compartment during natural T cell development. This therapy would give rise to long lived, natural Tregs with enhanced immunosuppressive function that can provide long term protection against autoimmune disease. To develop our Treg specific lentiviral construct, we first used a bioinformatic approach to identify 14 endogenous promoters and 30 enhancer elements that were preferentially active in human Treg cells. Candidate sequences were incorporated into our lentiviral vectors and screened *in vitro* in primary murine/human T cells to demonstrate their inherent specificity and activity. The addition of 6 tertiary regulatory elements were also then independently evaluated to maximise promoter performance.

In parallel, we designed a CAR with specificity for myelin oligodendrocyte glycoprotein (MOG), an autoantigen associated with MS. This aMOG-CAR was first expressed in Jurkat cells to assess tonic signalling and functionality in response to MOG antigen. It was subsequently confirmed that ligation of the aMOG-CAR on primary murine/human Tregs enhanced immunomodulatory function with significantly higher production of cytokines such as IL-10 compared to TCR stimulation. To establish a murine model of HSC gene therapy, murine lineage$^-$ HSCs were transduced with lentiviral constructs containing our aMOG-CAR under the control of our Treg specific promoter element and transplanted into conditioned animals. Following engraftment and reconstitution, CAR expression was assessed throughout the immune system to evaluate the biodistribution of CAR expression within the hematopoietic compartment. We demonstrate that such HSC-derived aMOG-CAR Tregs are stable, long lived and phenotypically normal. Furthermore, *ex vivo* CAR stimulation leads to enhanced Treg function with increased expression of CD25 and IL-10 secretion.

This work represents the first proof-of-concept study for the development of functional CAR-Tregs *in vivo* using HSC-GT. This targeted immunomodulatory approach provides a therapeutic strategy to overcome the significant limitations of other therapeutic modalities, such as autologous HSC transplantation and adoptively transferred CAR-Tregs, which do not result in long-term remission. Realisation of the potential of HSC-derived CAR-Treg for immunomodulatory therapies presents an opportunity to provide lifelong treatments for a broad spectrum of severe chronic autoimmune disorders including multiple sclerosis.

P0294

AAV-mediated gene therapy strategy for hereditary spastic paraplegia type 52 in hiPSC and mouse models

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Hereditary spastic paraplegia type 52 (SPG52) is an ultra-rare inherited neurological disorder characterized by weakness and spasticity in the lower limbs, global developmental delay, intellectual disability, and seizures. SPG52 is attributed to a loss-of-function mutation in the AP4s1 gene, responsible for encoding a subunit of the adaptor protein complex 4 (AP-4). Disruptions in any subunit of the AP-4 complex lead to its destabilization and degradation, resulting in a shared pathophysiology and symptomatology. While the exact function of AP-4 remains unclear, it is believed to have a role in the biogenesis of the autophagosome. Currently, there is no cure or treatment for this devastating condition. Gene therapy aimed at restoring AP4s1 expression represents a rational therapeutic approach for SPG52.

In order to establish a relevant human model of the disease and explore new potential therapeutic interventions, we generated induced pluripotent stem cells (iPSCs) from a skin biopsy of an SPG52 patient and two controls, a healthy carrier of the mutation and an age-match control. Cortical neurons differentiated from SPG52-iPSC showed accumulation of the AP-4 complex main cargo ATG9A within the Trans-Golgi network, abnormal neurite morphology, and deficient arborization, as well as a decreased activation of the neuronal network, which were not evident in neurons differentiated from Ctrl-iPSC. Upon delivery of a correct copy of the AP4s1 gene using AAV vectors, we are able to rescue disease-related phenotypes.

In parallel, we generated the first mouse model for SPG52 carrying a complete knock-out (KO) of the AP4s1 gene. KO mice show decreased body weight gain, impaired motor coordination, reduced strength, and a marked clasping phenotype. Electromyography showed decreased compound muscle action potential (CMAP) amplitudes of the hindlimb muscles confirming neuromuscular deficits. Moreover, AP4s1 KO mice exhibited hippocampal-related learning impairments, as well as a more active exploratory behavior without signs of anxiety. One of the main hallmarks of the disease, the thinning of the Corpus Callosum described in patients was also recapitulated, evidenced by magnetic resonance imaging, and subsequently validated through histological examination. Importantly, this model shows a significantly shortened lifespan compared to healthy controls. We intravenously administered neurotropic AAV vectors to deliver a correct copy of the AP4s1 gene in KO animals at two different ages, neonatal and young adults. We assessed the phenotype rescue of our approach through a range of motor tests and electromyography.

The development and treatment of hiPSC-derived cortical neurons and the AP4s1 KO murine model, both recapitulating SPG52 disease hallmarks, are crucial to validate and accelerate an *in vivo* gene therapy approach for SPG52 and other diseases affecting the central nervous system.

P0295

Rescue of auditory function in DFNB8 mice using a novel AAV capsid variant for efficient inner ear gene therapy

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Hearing loss affects over 460 million people worldwide, thereby significantly impacting quality of life. Genetic analysis has identified more than 150 causative monogenic genes for non-syndromic sensorineural hearing loss, presenting attractive targets for gene therapy interventions. Patients with TMPRSS3 gene mutations suffer from recessive deafness disorders termed DFNB8/DFNB10, with cochlear implantation being their only treatment option, though outcomes are often poor. Adeno-associated virus (AAV) vectors are being developed for inner ear gene therapy but require engineering to reach their full potential.

We report on the development of a potent capsid-engineered AAV vector, V6, which efficiently transduces inner and outer hair cells as well as spiral ganglion neurons across the entire cochlea already at low doses (1e6 per cochlea), as shown by 3D projections of vector distribution in the adult murine cochlea. DPOAE measurements pre- and post-vector delivery confirmed vector safety. Aiming to develop a treatment for DFNB8/DFNB10 patients, we used a knock-in mouse model carrying a common human DFNB8 TMPRSS3 mutation. These TMPRSS3$^{A306T/A306T}$ homozygous mice exhibit delayed-onset progressive hearing loss similar to human DFNB8 patients. Using our novel capsid variant V6 as a vector to deliver the human TMPRSS3 gene, a single V6-hTMPRSS3 injection into the inner ear of 6-month-old TMPRSS3$^{A306T/A306T}$ mice resulted in consistent TMPRSS3 expression in hair cells and spiral ganglion neurons. Five months post-injection treated ears showed overall lower hearing thresholds compared with untreated control ears at tested frequencies of 4, 8, 16 and 32 kHz, indicating treatment success, with significant reductions at 16 and 32 kHz. A comparison with the benchmark AAV2-hTMPRSS3 revealed that achieving the same level of hearing rescue required a 3-log fold higher concentration of the vector compared to V6-hTMPRSS3.

To further characterise V6, our novel capsid was compared to AAV-Anc80, a capsid engineered vector already in a human clinical trial for inner ear gene therapy, and AAV2. V6 demonstrated higher transgene expression in HEI-OC1 cells, an auditory cell line, compared to AAV-Anc80 and AAV2 (GOI 2000, 93 %, 29 % and 46 %, respectively), despite significantly lower entry efficiency, indicating enhanced intracellular processing. Subsequent IF-FISH analysis at single-cell level visualised V6 uncoating to approximately 4-fold higher levels than AAV2 after 24h. In contrast to AAV2, V6 uncoating appeared independent of nucleolar reorganisation, which has been shown to correlate with cell cycle progression for AAV2. This characteristic potentially offers an advantage for V6 in post-mitotic auditory cells. Furthermore, V6 outperformed AAV2 with 2-fold higher uncoating *in vitro* (HEI-OC1) in an indirect assay, correlating with corresponding transduction efficiencies.

In conclusion, our novel AAV vector V6 demonstrated effective and consistent inner ear cell transduction, likely driven by enhanced uncoating, and achieved successful hearing rescue in an aged mouse model of human genetic deafness at low doses. This lays the groundwork for developing V6-hTMPRSS3 gene therapy to treat DFNB8 patients.

P0296

AAV-mediated Delivery of Human APOE2 Variant APOE2-Christchurch Protects Against Neuronal Loss and Preserves Myelin Integrity in Humanized Tau Model of Alzheimer's Disease

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Knowledge that variants of the apolipoprotein (APOE) are the major genetic risk factors for Alzheimer's disease (AD; APOE3 average risk, E4 markedly increased risk, E2 decreased risk) led to the concept that genetic delivery of APOE2 to the central nervous system (CNS) of APOE4 homozygotes wold be therapeutic, preventing the APOE4-driven abnormal pathology that causes AD. This concept was supported by preclinical data in a murine humanized APOE4 model of AD demonstrating efficacy of CNS administration of AAVrh.10hAPOE2, an adenoassociated virus (AAV) serotype rh.10 coding for human APOE2 and led to LEXEO Therapeutics ongoing clinical trial of CNS administration of AAVrh.10hAPOE2 to APOE4 homozygotes with early AD (NCT03634007). The focus of the present study is to test the hypothesis that addition of the APOE Christchurch variant (Ch, R136S) to the APOE2 allele would create a 2nd generation vector that will enhance the efficacy of the therapy. This concept is based on the clinical observation that a woman with the dominant presenilin mutation was protected from the development of AD-related tau pathology by co-inheritance of APOE3Ch, i.e., despite genetic pressure to develop AD, co-inheritance of APOE Christchurch was protective (Arboleda-Velasquez JF et al, Nat Med 2019; 25:1680). Given the demonstrated therapeutic effects of AAV gene transfer of APOE2, we hypothesized that CNS delivery of the APOE2Ch variant would be more effective in protecting against Tau pathology in the P301S/TRE4 (human APOE4) mouse that exhibits biochemical and behavioral Tau-related features of AD. We administered AAVrh.10hAPOE2Ch to the hippocampus ($2.5x10^{10}$ viral particles, bilateral) at 5.5 months of age, a time where the mice already exhibit CNS Tau-related AD pathology. Behavioral, biochemical and genetic assessments were carried out at 8.5 months of age. Controls received either PBS or AAVrh.10Null without a transgene. Compared to AAVrh.10APOE2, mice treated with AAVrh10.hAPOE2Ch showed significant clinical improvement in behavioral deficits, assessed by nesting, y-maze, novel object recognition and Barnes maze tests (all $p<0.01$ compared to PBS and AAVrh.10Null). Immunohistochemistry and spatial transcriptomics were used to evaluate neuronal health. Compared to controls, AAVrh10.hAPOE2Ch significantly reduced neuronal and oligodendrocyte loss in the hippocampus (NeuN and Olig2 levels assessed by immunohistochemistry, $p<0.01$). Spatial transcriptomics revealed that AAVrh.10hAPOE2Ch significantly increased the expression of markers associated with neuronal health, myelin integrity and synaptic function (up to 5-fold difference compared to controls). qPCR and transcriptomic analyses also indicated significant decrease in damage-associated microglia markers and increase in homeostatic markers in the hippocampus, suggesting that AAVrh.10hAPOE2Ch corrected microglia-related pathology. In the AAVrh.10hAPOE2Ch treated group, tissue qPCR analysis also confirmed a significant reduction in pro-inflammatory markers ($p<0.01$) and increase in anti-inflammatory markers ($p<0.01$). In summary, addition of the Ch variant to the APOE2 variant was more effective than the APOE2 variant alone in correcting the AD Tau-related pathology, supporting the concept that AAVrh.10hAPOE2Ch represents a 2nd generation candidate to treat homozygous APOE4-associated AD.

P0297

Development and validation of an AAV gene therapy for mucopolysaccharidosis type IIIB in dog model of the pathology

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MPSIIIB is an autosomal recessive lysosomal storage disorder, caused by alpha-N-acetylglucosaminidase (NaGlu) enzymatic deficiency leading to accumulation of Heparan Sulfate Oligosaccharides (HSO) in tissues including the central nervous system (CNS). Patients manifest with early developmental delays followed by severe behavioral abnormalities, progressive neurodegeneration, and death before the age of 20 years. To date, there are no curative therapies for MPSIIIB. We have previously conducted a AAV-2/5 phase I/II intracerebral gene therapy trial that has shown promising results in four MPSIIIB patients with best results being obtained in the youngest patient (18 months-old). However, disease progression in tissues as important as meninges, brain capillary walls, and choroid plexus was presumably not stopped. Therefore, treatment of patients younger than 2 years and the delivery of NAGLU both within and outside the brain was concluded. We recently described and presented last year a novel AAV gene therapy using AAVPHP.eB-CAG-NaGlu vector. Here we are evaluating the therapeutic efficacy in the dog model of the pathology after combined intracerebral and intravenous delivery and data will be presented demonstrating a rescue of the enzymatic activity in the whole CNS. Safety data will also be presented as well as the ongoing study using a single IV delivery of MacPNS1 in order to prepare a phase I/II clinical trial submission to the EMA.

P0298

Multi-SINEUP: a novel RNA therapeutic approach for 22q11.2 microdeletion syndrome

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SINEUPs are a functional class of natural and synthetic antisense long non-coding RNAs that enhance the translation of partially overlapping sense mRNAs. Their activity depends on the combination of two domains: the overlapping region that dictates the specificity (binding domain, BD), and the embedded inverted SINEB2 element that acts as the effector domain (ED) controlling the enhancement of mRNA translation. By artificial engineering, synthetic SINEUPs can increase the translation of virtually any target gene of interest. Previous studies demonstrated the efficacy of SINEUPs *in vitro* and *in vivo* in enhancing the translation of the target gene. Given their functionality, among several possible applications, SINEUPs are potentially curative for many serious genetic diseases called haploinsufficiency, caused by mutations that inactivate one allele and lead to an insufficient amount of a gene product. Among them, there are cases of microdeletions of an entire portion of one of the homologous chromosomes leading to haploinsufficiency of multiple genes. One example is 22q.11.2 deletion syndrome, which manifests as multi-organs dysfunctions, ranging from cardiac defects to neuropsychiatric

symptoms. Therapeutic approaches that aim to restore the functions of a disrupted gene range from gene replacement therapy to RNA therapeutics. However, with the current knowledge, only one gene at the time is usually targeted thus leaving more complex diseases such as microdeletions without therapeutic options. In this study, we designed and synthesized the first multi-BD-SINEUP targeting multiple genes as a therapeutic strategy for 22q.11.2 deletions syndrome. By targeting TBX1, COMT and DGCR8, we demonstrated that the multi-BD-SINEUP could increase the protein levels of the three genes in vitro in cells and *in vivo* in mouse brain. Moreover, the multi-BD-SINEUP was able to rescue the cognitive impairments present in the LgDel mice, a mouse model of 22q11.2 deletion syndrome. In conclusion, we described the first multi-BD-SINEUP that could target and increase the translation of multiple mRNAs at the same time, with a proof-of-concept therapeutic application for 22q11.2 deletion syndrome.

P0299

Development and translation of a novel CRISPR/Cas13 RNA-editing therapy, HG204, to treat the neurodevelopment disease of *MECP2* duplication syndrome in HERO clinical trial

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Methyl-CpG binding protein 2 (*MECP2*) is a dosage-sensitive, X-linked gene critical for neurodevelopment whose gain-of-function causes *MECP2* duplication syndrome (MDS). This devastating disorder is characterized by severe intellectual disability, motor dysfunction, infantile hypotonia, epilepsy, respiratory tract infections, and premature death with no curative therapy. We investigated a translational strategy using CRISPR high-fidelity Cas13Y (hfCas13Y) to reduce the MeCP2 proteins and reverse disease phenotypes. Here, we developed HG204, consisting of hfCas13Y and *MECP2*-targeting gRNAs (g*MECP2*) packaged into a single AAV vector (AAV-hfCas13Y-g*MECP2*), then evaluated the editing efficacy in the brain of the *MECP2* transgenic (*MECP2*-TG) mice after neonatal intracerebroventricular (ICV) injection. The motor function, anxiety behavior, society impairment, and the restoration of fear learning deficits using the fear conditioning test of *MECP2*-TG mice were tested at 8 and 26 weeks following ICV injection. The long-term effects (48 weeks post-injection) and safety of AAV-hfCas13Y-g*MECP2* were further investigated by RNA-seq and RNA integrity analysis in the cortex tissues 4 and 48 weeks after ICV injection. Then, we plan to initiate an open-label and dose-escalation **HERO** (a first-in-**H**uman clinical trial to **E**valuate the safety, tolerability, and efficacy of a single ICV injection of HG204 **R**NA-editing therapy in MDS, a rare **O**rphan disease) study. We demonstrated HG204 editing efficacy in reducing MeCP2 proteins in the humanized *MECP2*-TG mice 8 and 26 weeks after ICV injection. The motor function, anxiety behavior, and society impairment of *MECP2*-TG mice were rescued by HG204. The fear conditioning tests revealed that 8-week-old *MECP2*-TG mice displayed an abnormal increase in fear learning in context paradigms, a hippocampal-dependent behavior, and was reduced by the HG204 at both 8 weeks and 26 weeks post-treatment. Notably, HG204-treated *MECP2*-TG mice had a significant increase in median lifespan (226 days) compared to that of PBS-injected littermates (156 days) ($p<0.01$). RNA-seq analysis showed that abnormal gene expression in *MECP2*-TG male mice was primarily restored. Cortical RNA integrity remained unaltered compared to PBS-injected controls at both time points,

suggesting that the hfCas13Y-g*MECP2* system lacked detectable collateral activity *in vivo*. H&E staining showed no injury in the brain, spine cord, liver, and kidney of *MECP2*-TG mice 48 weeks after treatment. Six 2 to 18 years of age (inclusive) boys with genetically confirmed MDS and stable pattern of seizures within 8 weeks before screening will be enrolled into the **HERO** study. The primary objectives are the safety and tolerability of HG204 at different doses. Secondary objectives include change from baseline in motor behavior, adaptive behavior, frequency of seizures, and other assessment scales within 52 weeks after the injection. Our preclinical findings demonstrate the long-term efficacy and safety of AAV-hfCas13Y-g*MECP2* treatment in *MECP2*-TG mice, supporting the clinical development of HG204 CRISPR/RNA therapeutic strategy for MDS treatment. HG204 has granted orphan drug and rare pediatric disease designations by the U.S. FDA and orphan drug designation by EMA.

P0300

CSF-directed administration of an AAV9-*ASAH1* vector to prevent acid ceramidase deficiency in newborn P361R-Farber mice

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Farber disease (FD) and spinal muscular atrophy with progressive myoclonic epilepsy (SMA-PME) are autosomal recessive disorders resulting from mutations in the *ASAH1* gene, which encodes a ubiquitously expressed lysosomal enzyme, acid ceramidase (ACDase). This enzyme catalyzes the conversion of ceramide, a bioactive lipid, into sphingosine and fatty acid. Mutations affecting ACDase activity promote the toxic accumulation of ceramides, leading to the infiltration of inflammatory cells and lesions in both central nervous system and peripheral tissues. Currently, there is no curative treatment, highlighting a significant unmet medical need. In the present study, we evaluated a gene therapy approach in a severe mouse model of acid ceramidase deficiency by delivery of an AAV9 vector expressing human ACDase into the cerebrospinal fluid (CSF). We report that intracerebroventricular administration of the vector in newborn FD mouse model (*Asah1*$^{P361R/P361R}$, P361R-Farber) prolonged the lifespan of animals, improved body growth and enhanced motor activity. While the treatment effectively mitigated central nervous system symptoms, it did not fully address peripheral organ manifestations. The degree of phenotype correction correlated with vector biodistribution and transgene expression in tissues. These results suggest that CSF-directed delivery of an AAV9-*ASAH1* vector may represent a therapeutic strategy to primarily address the central nervous system clinical manifestations of acid ceramidase deficiency, as seen in patients affected by SMA-PME.

P0301

Precision neuromodulation of neural circuit activity to treat prevalent disorders

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Neural circuits throughout the central and peripheral nervous system play an important role in many disease states. Normal function of these circuits depends on coordinated electrical activity, and aberrant activity can cause debilitating symptoms and disease. We are developing a new generation of gene therapies that target specific neural circuits and provide a mechanism for modulating activity in a way that could be controlled by the patient and physician. Our precision gene therapy has two key components: (a) a targeting vector system to selectively access dysfunctional circuits; and (b) a controllable therapeutic system to increase or decrease neuronal activity through an easily administered (e.g. oral) medication based on the needs of the patient. Sania Therapeutics is developing precision gene therapies that combine cell-type-specific AAV vectors with delivery of a protein that enables controllable neuromodulation. This approach will allow us to selectively modulate neural circuit activity in disease states while minimizing adverse effects associated with current therapies. From a therapeutic perspective, the ideal neuromodulation system would have several properties: i) minimal effects on baseline neuronal activity in the absence of an activator; ii) potent and titratable effects on neuronal activity in response to an activator; (iii) activated by a drug that can be readily administered orally and has minimal effects on non-expressing cells; and iv) validated efficacy in humans. Here we present data showing the efficacy of two neuromodulation systems, SRx-C490 and SRx-C500, for modulating excitability of human iPSC derived motor neurons (MNs) *in vitro*, and in a mouse model of motor neuron hyperexcitability *in vivo*. Motor neuron hyperexcitability is the underlying cause of spasticity, a prevalent disorder characterised by uncontrollable muscle contractions and without any effective treatment options. SRx-C490 had no effect on baseline activity (no activator) in single MNs or on network activity at the population level, however administration of the activator induced dose-dependent reductions in activity for SRx-C490 transduced MNs at nanomolar doses, a range at which control MNs (AAV-GFP) were unaffected. We next assessed the efficacy of SRx-C490 for reducing activity in hyperexcitable MNs in a mouse model of spasticity. SRx-C490 was expressed in MNs following injection into spastic muscles, via retrograde transport, but had no baseline effect on MN activity or spasticity. However, spasticity was significantly reduced when the activator was administered at 2mg/kg. By introducing mutations into SRx-C490, we created a more potent inhibitory channel, SRx-C500, which enabled the activator to reduce spasticity at doses at least 4x lower than SRx-C490. In summary, we show that AAV-delivered SRx-C490 is effective at reducing excitability in human MNs *in vitro* and alleviates spasticity in mice when activated by an FDA approved drug. Further, we can increase the efficacy of our system with rational mutations to our channel. These novel neuromodulation systems, in combination with Sania's cell-type specific AAV vectors, highlight the potential of our precision gene therapies to treat millions of patients across a broad range of disease areas.

P0302

Muscle-targeted α-Klotho outperforms CNS delivery in ALS treatment efficacy

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Amyotrophic lateral sclerosis (ALS) is characterized by progressive muscle weakness due to axonal detachment from muscles and motoneuron (MN) loss. To preserve neuromuscular function in the SOD1^{G93A} mouse model, we targeted α-Klotho (KL) to either skeletal muscles or the spinal cord at an early disease stage. KL overexpression in skeletal muscles was achieved using systemic MyoAAV vectors and the desmin promoter. For spinal cord targeting, KL was delivered via lumbar intrathecal administration of AAV9 vectors using the CMV promoter.

The first approach improved motor function, delayed disease onset, and maintained muscle mass. Enhanced neuromuscular function was evidenced by increased compound muscle action potential (CMAP) amplitudes and more functional hindlimb motor units compared to controls. Additionally, KL-treated mice showed preserved motor evoked potentials (MEPs), indicating improved connectivity between corticospinal and spinal MNs. Muscle overexpression of KL also preserved the innervation of motor endplates, protected MNs, and extended the lifespan of treated SOD1^{G93A} mice compared to controls. Conversely, central nervous system (CNS) overexpression of KL had a milder impact on neuromuscular preservation. CMAP and MEP amplitudes and muscle mass were only preserved in SOD1^{G93A} KL-treated females. However, motor function was improved in both sexes, despite not observing benefits in SOD1^{G93A} male mice for other parameters. Thus, targeting KL to skeletal muscles is a more effective approach for promoting functional improvement in this ALS mouse model.

To understand KL's protective mechanisms in our muscle-targeted therapy, RNA sequencing and proteomic studies were conducted on the lumbar spinal cord and gastrocnemius muscles. Spinal cord profiling revealed that KL secretion enriched synaptic activity and plasticity, reduced inflammatory and oxidative responses and decreased mitochondrial and apoptotic alterations in SOD1^{G93A} mice. In the muscles, KL overexpression significantly restored gene expression patterns, particularly in pathways related to muscle contraction and development, and inhibited fibrosis and atrophy. In both tissues, novel roles for KL in promoting ubiquitin-mediated proteolysis and correcting RNA disturbances were discovered.

Overall, our findings demonstrate that muscle-targeted KL secretion, rather than intrathecal delivery and spinal cord overexpression, can protect against ALS progression in the SOD1^{G93A} model. This study identifies new roles for KL and offers insights for future interventions in ALS and other neurodegenerative disorders.

P0303

TREM2 microglia engineering via HSC gene therapy – an innovative treatment approach for Alzheimers' disease

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Alzheimer's disease (AD) is the most frequent form of dementia among the elderly. AD results from complex pathogenic events, including the deposition of extracellular amyloid-β (Aβ)-containing plaques that are considered as central players in initiating and sustaining the neurodegenerative process. TREM2 (Triggering receptor expressed on myeloid cells 2) is a microglia cell-surface receptor whose deficiency or haplo-insufficiency augments Aβ accumulation in AD brain due to a dysfunctional response of microglia cells, which become apoptotic. Indeed, TREM2 binds to apolipoproteins, including APOE and CLU/APOJ, and thereby facilitates uptake of Aβ by microglia. Carriers of TREM2 hypofunctional variants are for this reason exposed to an increased risk of developing early onset AD.Based on these observations, we predict that raising and restoring TREM2 expression in the brain of AD patients, and in particular in their microglia, would result in therapeutic benefit. Our working hypothesis is thus that intra-CNS transplantation of Hematopoietic Stem Progenitor Cells (HSCs) engineered by Lentiviral vectors (LVs) to express robust TREM2 levels in response to tissue damage in their myeloid/microglia cell progeny would modulate neuroinflammation, restore physiological microglia functions and contribute preventing and reducing Aβ accumulation in the CNS of AD patients.

We have already conducted a *Proof of Concept (Poc)* in-vivo study in a relevant Alzheimer's Disease mouse model (5XFAD mice) with an age dependent and progressive Aβ accumulation which recapitulates main clinical manifestations found in patients. We employed a 3rd generation LVs expressing the human TREM2 cDNA under the control of the human phosphoglycerate kinase (hPGK) promoter and an optimized protocol for the directed and CNS-restricted intracerebroventricular administration of TREM2 expressing HSPCs from 5XFAD donor mice into myeloablated AD recipients. Gene therapy substantially alleviated the memory deficits in an alternation task and at the Morris Water Maze test, and reduced anxiety levels measured at the Elevated Plus Maze test, especially at late times points of observation when treatment halted the progression of the pathology comparted to controls groups. Histopathological postmortem analysis showed reduction of microgliosis and neuroinflammation, and an overall reduced Aβ accumulation in the CNS of the treated animals with transplant-derived TREM2-expressing cells clustered around Aβ plaques in the brain parenchyma. These results are being integrated with equivalent gene therapy strategy with a novel inducible synthetic promoter able to safely deliver multiple copies of the human TREM2 cDNA in 5XFAD model l to generate additional data in support of treatment indication, being, in this way, further refined by employing the new regulated myeloid promoter to enable a faster path towards clinical translation.Overall, these data provide first proof of therapeutic potential of HSPC gene therapy in a pure neurodegenerative condition as well as first evidence of therapeutic benefit associated to a CNS-restricted engraftment of engineered hematopoietic cells.

P0304

CRISPR/Cas13 RNA-targeting of *Bace1* rescues memory deficits in Alzheimer's disease

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Alzheimer's disease (AD) is a progressive neurological disease and the most common form of neurodegenerative dementia manifesting as cognitive deficits and amyloid beta (Aβ) plaques and neurofibrillary tangles in the brain, resulting in irreversible neuronal death, memory loss, apathy, depression, and irritability. The currently available therapeutic options are merely symptomatic and supportive, with side effects such as confusion, dizziness, depression, constipation, and diarrhea. There is no specific disease-modifying treatment, which brings a significant burden to the family and society. β-secretase BACE1 (β-site amyloid precursor protein cleaving enzyme) is considered the potential and rationale target because it is involved in the rate-limiting step, which produces toxic amyloid β (Aβ) peptides that lead to deposits in the form of amyloid plaques extracellularly, resulting in AD. Here, we developed CRISPR/high-fidelity Cas13Y (hfCas13Y) RNA-targeting therapy for AD**,** precisely and efficiently targeting the *BACE1* mRNA. We first screened the gRNAs of *BACE1* (g*BACE1*) by fluorescence-activated cell sorting (FACS) in 293, N2a, and Cos7 cells. Then, we administrated doses of AAV-hfCas13Y-g*BACE1* into the hippocampus of wild-type C57/B6 mice through stereotactic injection and assessed the knockdown efficiency through qPCR, Western Blot (WB) and immunostaining at 2- and 8-week post-injection. To evaluate the therapeutic efficacy, we stereotactically administrated the AAV-hfCas13Y-g*BACE1* into both hippocampal regions of 4-month-old 5xFAD mice, a widely-used mouse model of AD. Fear conditioning, Barnes, and water maze tests were conducted to evaluate the learning and memory capabilities of the 5xFAD mice 3- and 6-month post-injection. The qPCR, WB, immunofluorescence (IF) staining, and enzyme-linked immunosorbent assay (ELISA) analysis were performed to measure Bace1 and Abeta levels. Electron microscopy (EM) was utilized to analyze the number of synapses. Moreover, we systematically delivered AAV-hfCas13Y-g*BACE1* to 5xFAD mice by retro-orbital injection. We evaluated the treatment's efficacy through the abovementioned behavior tests, qPCR, WB, IF staining, ELISA analysis, Golgi staining, and EM. We have developed novel CRISPR/hfCas13Y systems and identified two gRNAs that efficiently target the *Bace1* mRNA in humans (reduced 99% in 293 cells, $p < 0.001$), monkeys (reduced 98% in Cos7 cells, $p < 0.001$) and mice (reduced 92% in N2a cells, $p < 0.01$) cells. Then, we evaluated the *in-vivo* knockdown efficiency of the two AAV-hfCas13Y-*BACE1*-gRNAs at different doses in C57 mice and observed that AAV-hfCas13Y-*BACE1*-gRNA2 demonstrated superior performance by reducing *Bace1* mRNA levels nearly 50% ($p < 0.01$). Upon injection of AAV-hfCas13Y-*BACE1*-gRNA2 into the hippocampus of 5xFAD mice, we also observed a reduction in Bace1 expression. Furthermore, we systematically administered AAV-hfCas13Y-*BACE1*-gRNA2 via retro-oral injection. The results from fear conditioning and water maze tests indicated that the learning and memory capabilities of hfCas13Y-*BACE1*-gRNA2 treated 5xFAD mice were enhanced when compared to hfCas13Y-HA treated 5xFAD mice. Our findings showed CRISPR RNA-targeting therapy efficiently reduced the *Bace1* mRNA expression, alleviated Abeta pathologies, improved neuronal functions, and rescued the learning and memory abilities in the mouse model for AD, suggesting that CRISPR/hfCas13Y RNA-targeting therapy is a potential approach for AD and may expand to other neurodegenerative diseases.

P0305

Scn1a gene upregulation mediated by artificial transcription factors as a treatment for Dravet Syndrome

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Dravet syndrome (DS) is a severe developmental and epileptic encephalopathy characterized at onset by drug resistant febrile and afebrile seizures, later often accompanied by developmental delay, cognitive impairments and behavioral deficits. About 20% of patients die before adulthood mainly due to Sudden Unexpected Death in Epilepsy (SUDEP). DS is caused by haploinsufficiency of *SCN1A* gene, encoding for the *alpha* subunit of the $Na_V1.1$ voltage-gated sodium channel, mostly in interneurons. Current pharmacological treatments are ineffective and novel therapies are urgently needed. Classic gene supplementation approaches are unsuitable due to the large *SCN1A* coding sequence, of about 6kb, which exceed the cargo capacity of available Adeno Associated Viral (AAV) vectors. An activatory CRISPR/dCas9-based approach has been already developed to boost *Scn1a* expression; however, some hurdles such as the possibility of immunological response and the necessity to use two distinct AAV vectors to deliver it, are still present. Here we exploit Zinc-Finger Proteins (ZFPs) fused to a VP64 transcriptional activator domain, to be used as artificial transcription factors of eukaryotic origin and fitting in a single AAV vector.

We designed 12 ZFPs targeting *Scn1a* gene promoter and identified some of them able to upregulate *Scn1a* mRNA and $Na_V1.1$ protein levels of about 2 and 1.5-folds, respectively, in mouse primary neurons. A subset of ZFPs targeting a regulative region conserved between mouse and human species were tested in human Neural Progenitor Cells (NPCs)-derived neurons, where their ability to upregulate *SCN1A* mRNA level was confirmed. One selected activatory ZFP was V5 tagged and cloned under a constitutive promoter to produce an Adeno-Associated Viral vector (AAV). ZFP-AAV was delivered *in vivo* by intracerebroventricular injection (ICV) in perinatal Dravet mice. AAV transduction efficiency was assessed by quantification of V5 positive cells over the total NeuN positive, showing that 70% and 80% of neurons were transduced in the cortex and hippocampus, respectively. Additionally, Dravet mice injected with activatory ZFP-AAV displayed an amelioration of SUPED incidence, decreasing from 40% to 20% in mutant injected mice. A reduced susceptibility to thermal induced seizures was also observed, with an increase in the temperature of induction of about 2 C° with respect to control conditions.

In conclusion, our *in vitro* data suggest the efficacy of activatory ZFPs treatment in boosting *Scn1a* gene transcription both on mouse primary neurons and on human NPCs derived neurons. Additionally, their delivery *in vivo* by AAV vector ensures a good transduction efficiency together with an amelioration of DS phenotypic manifestations.

P0306

Gene therapy rescues white matter swelling and motor impairment in a mouse model of megalencephalic leukoencephalopathy with subcortical cysts (MLC)

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Megalencephalic leukoencephalopathy with subcortical cysts (MLC, also known as Van der Knaap disease) is an ultrarare infantile onset leukodystrophy. Over 70% patients bear biallelic loss-of-function mutations in *MLC1* and present with early-onset macrocephaly, cerebellar ataxia, spasticity and muscle stiffness, epileptic seizures, and mild cognitive decline with or without autism. These symptoms correlate with progressive vacuolation of astrocytic endfeet and myelin sheaths, leading to white matter oedema and the development of subcortical cysts. *MLC1* encodes an integral oligomeric protein that is specific to the astrocytic lineage and is primarily found at the Bergmann glia, perivascular astrocytic endfeet, and astrocyte-astrocyte cell junctions, where it acts as an interaction hub for a myriad of water and ion channels, G protein-coupled receptors and other membrane-bound proteins. *MLC1* mutations found in patients reduce protein stability and compromise the regulation of this complex interaction network, leading to unbalanced water and ion homeostasis.

Our objective is to develop a gene therapy strategy for MLC using the *Mlc1*-/- mouse model and to identify new biomarkers that can be eventually used to validate its therapeutic efficacy. As a proof of concept, we intravenously administered AAVs able to cross the murine blood-brain barrier to deliver a correct copy of the human *MLC1* cDNA, under the regulation of the human astrocyte-specific promoter gfa2 to 10-month-old *Mlc1*-/- mice, 7 months after white mater vacuolation onset. One year after gene therapy, in vivo magnetic resonance imaging (MRI) revealed a normalization of brain water content and oedema and white matter microstructure in treated *Mlc1*-/- mice, two essential parameters altered in MLC patients. Histopathological analyses confirmed full rescue of white matter vacuolation and MLC1 localization in Bergmann glia and blood vessels, validating MRI findings. Importantly, we detected robust astrocyte-driven expression of MLC1 as later as one year after viral vector administration. On the other hand, gene therapy successfully reversed the progression of motor impairment in treated mice, as evidenced by behavioural tests used for assessing cerebellar ataxia-like symptoms. All in all, our results not only demonstrate the sustained efficacy of our preclinical gene therapy, but also underscore the reversibility of white matter vacuolation and motor impairment, suggesting a broad therapeutic window for therapeutic intervention in MLC patients.

P0307

A novel approach for treatment of rett syndrome and other x-linked disorders through miREX gene regulation combined with Falcon™ precision CSF delivery

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X chromosome inactivation is a developmentally regulated process in females, wherein one X chromosome per cell is inactivated through a complex stepwise process involving the Xist RNA and many other factors. This process allows for dosage regulation in females for X-linked gene expression. As a consequence, female tissue affected by X-linked genetic disorders, such as Rett Syndrome, contain a mixture of cells expressing the mutated version of the gene or the healthy copy depending on which X chromosome is active in any given cell. This mosaicism complicates gene replacement strategies significantly as the healthy cells are at risk of being overdosed. Notably, all cells that express the mutated version of the protein contain a healthy gene copy on the silenced chromosome which, upon re-expression, could ameliorate the disease phenotype. Thus, mechanisms that regulate X-inactivation may be an effective strategy for Rett Syndrome and other X-linked disorders. Here, we show that microRNA 106a leads to the stabilization of the long noncoding RNA, Xist, resulting in the inactivation of the X chromosome. Sequestration of miR106a using a sponge (miREX) leads to partial X chromosome reactivation (XR) and a subsequent increase in MeCP2 expression. Adeno-associated viral (AAV) vector based delivery of miREX to severe female Rett syndrome mice ameliorated clinically relevant behavioral phenotypes including hind limb clasping, tail lesions and abnormal respiration. In addition, miREX also rescued structural deficits in Rett patient derived neurons with a broad spectrum of MeCP2 mutations. We further confirmed efficacy in patient cell lines of three additional neurological conditions, underlining the ability to use miREX as a platform gene therapy for multiple disorders. Importantly, optimizing the targeting of the central nervous system is critical to ensure best therapeutic benefit for any CNS disorder including Rett Syndrome. Alcyone Therapeutics has developed a novel precision delivery platform that includes extensive in silico and in vitro modeling combined with in vivo validation in large animals (Falcon™). We demonstrated superior AAV9 biodistribution in terms of uniformity, deeper brain structure penetration, and reduction of off-target effects in nonhuman primates using Falcon™. In summary, our miREX gene regulation platform is highly promising for treatment of Rett Syndrome and several other X-linked disorders. The safety and potential for efficacy is further enhanced by the use of a novel delivery technology (Falcon™) allowing reduction of off-target delivery and improving biodistribution in the brain.

P0308

Genome-edited hematopoietic stem cells and optimized brain conditioning enhance treatment efficacy for progranulin deficiency

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Hematopoietic stem cell transplantation (HSCT) can deliver therapeutic proteins to the central nervous system (CNS) through transplant-derived microglia-like cells, which replace microglia upon brain conditioning. This approach represents a promising therapeutic modality for fatal diseases caused by the deficiency of progranulin (GRN): neuronal ceroid lipofuscinosis type 11 (CLN11) or frontotemporal dementia (FTD-GRN), depending on the gene dosage. These diseases are particularly suitable for an HSCT-depot approach, given that GRN is a secreted lysosomal protein amenable to cross-correction of affected cells, such as neurons.

We investigated the efficacy of HSCT in correcting GRN deficiency in preclinical studies of HSCT. We observed that intravenous transplantation of wild-type hematopoietic stem and progenitor cells (HSPCs) in immunocompetent *Grn-/-* mice, conditioned using a novel protocol that combines busulfan and a 6-day course of PLX3397, resulted in efficient microglia replacement (91±2.6% of brain CD45+ cells). This approach partially corrected biochemical GRN deficiency in the brain and eyes and normalized brain lipofuscin storage, proteostasis, and lipid metabolism.

To develop an autologous HSCT for CLN11/FTD-GRN, we genetically modified mouse and human HSPCs by genome editing *ex vivo* to achieve the targeted integration of human GRN expression cassettes at safe harbor *loci* via homology-directed repair. Our candidate approach resulted in 30±3% and 39±3% edited alleles in mouse and human HSPCs, respectively. Transplantation experiments in immunocompetent and immunodeficient *Grn-/-* mice showed long-term engraftment of genome-edited HSPCs (GE-HSPCs) and multilineage differentiation capacity. Supra-physiological GRN expression and secretion from GE-HSPCs were achieved *ex vivo* and *in vivo* using strong non-viral promoters, and were not associated with detectable toxicity, supporting the therapeutic potential and safety of the approach. Efficient engraftment of mouse GE-HSPCs in immunocompetent *Grn-/-* mice receiving optimized brain conditioning corrected GRN deficiency and pathological hallmarks in the CNS.

Our work demonstrates the therapeutic potential of both allogeneic and autologous HSCT using genome-edited HSPCs to treat GRN deficiency. Therapeutic efficacy can be enhanced through genetic engineering and optimized CNS conditioning. Our results support the clinical development of these approaches for CLN11 and FTD-GRN.

P0309

Lentiviral *ex vivo* autologous HSC gene therapy as a tool to deliver therapeutic antibodies beyond the blood brain barrier

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Hematopoietic stem cell gene therapy (HSC-GT) allows potentially lifelong reconstitution of the immune system with cells that have been genetically modified *ex vivo* by lentiviral transduction to express a specific therapeutic transgene. Upon myeloablative conditioning, cells derived from transplanted HSCs are able to migrate and engraft into several organs, including the brain where they differentiate into microglia-like cells. These cells can then deliver therapeutic transgene expression directly within the central nervous system (CNS), overcoming the limitation of other therapeutic modalities such as enzyme replace therapies (ERTs), which fail to efficiently cross the brain barrier (BBB). The highly efficient capacity of HSC-GT to achieve engraftment of gene modified cells in the CNS has been clinically demonstrated in devastating neurometabolic disorders such as metachromatic leukodystrophy (MLD), where HSC-derived microglia-like cells restore Arylsulfatase-A enzyme expression directly in the CNS, preventing neurodegeneration and associated severe neurological defects in patients. Antibodies are effective therapeutic agents for a variety of pathologies. However, when delivered systemically, antibodies only minimally penetrate the BBB, and therefore have very limited efficacy in the treatment of CNS disorders. Here, we sought to investigate whether *ex vivo* autologous HSC-GT could provide an effective approach to deliver antibodies directly in the brain via expression by *in situ* engrafted gene-modified microglia-like cells.

We used a human microglia cell line (HMC3) and an optimised method for differentiation of human $CD34^+$ HSC into microglia cells (~90% $IBA1^+$ cells), for use as *in vitro* models to assess the capacity of this cell type to produce antibodies. We designed lentiviral (LV) vectors to express model single chain variable fragment (scFv) antibodies, such as anti-PD-1, under the control of different promoters. We demonstrate that microglia cells differentiated from LV transduced HSCs and transduced HMC3 cells can efficiently express and secrete scFv antibodies. We also confirmed the specificity and functional activity of microglia-secreted antibodies using *in vitro* mixed lymphocyte reactions to assay T cell activity. Using a murine model of HSC-GT, where gene-modified mouse $lineage^-$ HSCs were transplanted into mice conditioned with busulfan, we also confirmed that antibody production was detectable *in vivo* within the CNS tissue upon HSC engraftment and differentiation into microglia-like cells, and also blood plasma, where levels of up to 300ng/mL are achieved, corresponding to ranges of anti-PD1 levels observed in murine models of cancer immunotherapy. To further enhance HSC-derived microglia engraftment in the CNS by HSC-GT, we have evaluated the use of CSF1R inhibitors (PLX3397) in combination with total body irradiation, where addition of PLX3397 results in 6-8 fold higher levels of HSC-derived microglia engraftment (60-80% gene-marked microglia in CNS). Our ongoing studies are evaluating the improvements in levels of therapeutic antibodies that can be generated in the CNS by optimised engraftment of gene-modified microglial-like cells.

Microglia cells play a central role in neuroinflammation and they are particularly enriched in certain pathologies. The ability to harness their localisation in the CNS for the targeted delivery of therapeutic antibodies by HSC-GT could dramatically improve the prognosis of serious neurological conditions such as glioblastoma and neurodegenerative disorders.

P0310

Dual REVeRT AAV vectors lead to high expression of the full-length MYO7A protein in different species and in a mouse model for Usher syndrome 1B

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Usher syndrome is the most common form of inherited deaf-blindness. Mutations in *MYO7A* are the predominant cause for USH1B, the most severe subtype of Usher syndrome. There is currently no therapy that can halt or mitigate retinal degeneration in USH1B patients. As the *MYO7A* coding sequence (6.7 kb) exceeds the packaging capacity of adeno-associated-viral (AAV) vectors, alternative strategies including dual AAV approaches are required for gene supplementation. Here, we employed our recently published dual AAV vector mRNA *trans*-splicing approach (REVeRT) to deliver *MYO7A* in mice, pigs and human retinal organoids. Upon subretinal injection, we show that our dual REVeRT AAV vectors lead to high *MYO7A* reconstitution efficiencies in the mouse and pig retina at mRNA and protein level. A side-by-side comparison of REVeRT AAVs to a commonly used dual AAV approach based on reconstitution at the genomic level shows a 3-fold increase in *MYO7A* expression at transcript and 38% higher expression at protein level. The transgenic *MYO7A* protein was found to be mainly localized to the RPE and photoreceptor cells of mice, pigs and human retinal organoids. Lastly, we were able to restore the retinal *MYO7A* expression in a conditional Myo7a-KO-mouse-model to wildtype levels indicating the high therapeutic potential of this approach. Further pre-clinical experiments focusing on the melanosomes distribution in the RPE of Myo7a deficient animals after treatment are currently under investigation.

P0311

Targeting pathogenic Lafora bodies in Lafora disease using gene therapy

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Lafora disease (LD) is a rare and fatal neurodegenerative disorder, inherited in an autosomal recessive manner, presenting with progressive myoclonic epilepsy emerging in previously healthy adolescents. Dementia, ataxia, and dysarthria progress rapidly and intractable epilepsy develops, leading to death within 5-15 years from initial presentation of symptoms. To date no specific therapies exist. Two are the genes known to be responsible for LD, *EPM2A*, encoding laforin, a dual-specificity phosphatase, and *EPM2B*, encoding malin, an E3-ubiquitin ligase. Laforin and malin work together in an enzymatic complex that control glycogen synthesis. The absence of an active complex leads to the formation of Lafora bodies (LBs), that contain abnormal, insoluble, and hyperphosphorylated forms of glycogen, called polyglucosans. LBs drive the pathology, leading to neurodegeneration in LD. LBs form in neuronal perikarya, in neuronal short processes and in astrocytes. Extraneurally LBs form in heart, liver, and skeletal muscle, but they don't cause disease in these organs, at least during the short life of the patients. LBs were therefore identified as a target for ameliorating LD. Data from the literature highlights the role of human pancreatic alpha-amylase in degrading LBs *in vitro* and also *in vivo*, in a mouse model of LD. However, there are at least two relevant limitations for the use of enzymatic therapy in humans: the first being the necessity to inject the enzyme by intrathecal or intracerebroventricular routes, since the enzyme doesn't pass the blood brain barrier, and the second the necessity to repeat the infusion many times during the life or to use implantable pumps. To overcome these limits, we generated a new gene therapy approach for LD. We produced a plasmid containing the human alpha-amylase gene (*AMY2A*), regulated by a strong and ubiquitous CAG promoter, and we demonstrated, *in vitro* in wild type murine astrocytes, its ability to induce an active alpha-amylase expression. We then transfected murine astrocytes cultures taken from *Epm2b*$^{-/-}$ mouse model (MKO), that naturally accumulate LBs. We observed that the transfected astrocytes produced high levels of alpha-amylase, and that it reduced drastically the glycogen content. In particular, alpha-amylase reduced, in 48 hours, the glycogen load of the MKO astrocytes of about 80%, respect to not treated MKO astrocytes. We also demonstrated that alpha-amylase doesn't affect the viability of the transfected astrocytes. We then generated an AAV.PHP.eB vector containing the plasmid with human alpha-amylase gene and we started with intravenous injection into an *Epm2a*$^{-/-}$ *mouse* model of LD at one month of life. First, we evaluated the biodistribution of the virus using a GFP signal and we also conducted immunohistochemical, PAS staining and qPCR experiments to follow up the production of alpha-amylase in all brain areas. We then evaluated, *in vivo*, sacrificing the mice at 6 months of life, the ability of alpha-amylase to reduce LBs in the mouse brain. Experiments on motor coordination, object recognition task, sensitivity to PTZ, video EEG and electrophysiological experiments are ongoing, to verify the clinical effect of the gene therapy.

P0312

AAV-delivered *GRIN1* gene improves synaptic NMDA receptor function in mouse models of *GRIN* disorder

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N-methyl-D-aspartate receptor (NMDAR) is an ionotropic glutamate receptor that plays an important role in brain development, learning and memory. NMDARs are encoded by *GRIN* genes (*GRIN1*, *GRIN2A-D*, and *GRIN3A-B*). Rare *de novo* mutations in one of the *GRIN* genes lead to *GRIN* disorder, a neurodevelopmental disorder with symptoms of profound intellectual disability, epilepsy, and visual cortical impairment. Most pathogenic variants are missense heterozygous mutations that leads to over- or under-activation of NMDAR. As a result, pharmacological interventions directly targeting NMDAR often show mixed results in patients. Development of gene-based therapies are desired. A functional NMDAR is a heterotetrametric complex consists of two obligatory GluN1 subunits (encoded by *GRIN1* gene). The other two subunits are a combination of any of the GluN2 or GluN3 subunits. We hypothesize that delivery of exogenous *GRIN1* gene is beneficial for missense *GRIN1* variants. The exogenous *GRIN1* gene will lead to excess expression of functional 'wildtype' GluN1 proteins that competes with variant proteins for assembly into NMDARs. As a result, more NMDARs will contain only wildtype GluN1 subunits and function normally. To test our hypothesis, we developed two genetic constructs. Both constructs contain i) human *GRIN1* cDNA of the GluN1-1a isoform (hGRIN1); ii) a V5 tag sequence at the N-terminus; iii) 5' and 3' inverted terminal repeats (ITRs) for AAV transfer. We used two promoters that drive ubiquitous (hGAPDH) or neuron-specific (hSYN1) expression. The constructs are packaged into an AAVHSC capsid that can cross the blood brain barrier and transduce neurons and glial cells. We generated two mouse models possessing the equivalent heterozygous missense *GRIN1* patient variants, the Grin1$^{Q536R/+}$ and Grin1$^{Y647S/+}$ mice. Electrophysiological studies revealed that, remarkably, AAVHSC-delivered hGRIN1 showed significantly improved NMDAR-mediated synaptic transmission and restored long-term potentiation (LTP) in the hippocampus of Grin1$^{Q536R/+}$ mice. Importantly, the treatment did not negatively impact normal synaptic functions. Preliminary data suggest that similar rescue of NMDAR function is also observed in Grin1$^{Y647S/+}$ mice. For systemic transduction, we also administered the vectors intravenously (i.v.). hGRIN1 expression was detected at mRNA and protein levels in the brain of Grin1$^{Q536R/+}$ mice four- and twenty-week after i.v. injection. Efforts are made to determine the optimal dose, promoter, and route of administration for behavioural rescue. In summary, our strategy has translational potential for clinical applications and may serve as a novel one-time treatment for patients with pathogenic *GRIN1* variants.

P0313

CRISPR/hfCas12Max-mediated DNA-editing therapy improves motor coordination and neuromuscular function and significantly extends lifespan in amyotrophic lateral sclerosis mouse model

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The hallmark of amyotrophic lateral sclerosis (ALS) is the degeneration of the upper (cortical) and lower (spinal and brain stem) motor neurons, resulting in progressive muscular weakness, paralysis, and ultimately death within 2-5 years of symptom onset, mainly due to the respiratory failure. 97% of ALS patients exhibit cytoplasmic aggregation of mutated nuclear transactive response DNA-binding protein 43 (TDP-43), an RNA-binding protein essential for normal functions of the motor neurons, contributing to neuron death. Ataxin-2 is implicated in the

formation of stress granules containing TDP-43. Knockdown of Ataxin-2 has been shown to prevent the aggregation of TDP-43 stress granules. Here, we developed a potential therapeutic strategy using a high-fidelity Cas12Max (hfCas12Max) system, an engineered Cas12i with high activity and specificity, to target *ATXN2* as a novel treatment for ALS. We generated a single adeno-associated virus (AAV) vector packaging hfCas12Max and gRNA targeting *ATXN2/Atxn2* (g*ATXN2*) to target neurons in the brain and spinal cord when intracerebroventricularly (ICV) injected into the neonatal humanized *TDP-43*$^{Tg/Tg}$ mice, a mouse model of ALS. These mice display abnormal gait, severe tremors, and motor function decline, and they develop increasingly severe kyphosis, which typically begins manifesting at postnatal 14 days (P14). To monitor the effects of the treatment, we observed these phenotypes tri-daily between P15 and P21, including weight, which is also adversely affected in *TDP-43*$^{Tg/Tg}$ mice. The lifespan of mice injected with AAV-hfCas12Max-g*ATXN2* or PBS was observed daily. The editing efficiency of *Atxn2* DNA in the cortex and spinal cord of *TDP-43*$^{Tg/Tg}$ mice was detected by next-generation sequencing (NGS) analysis 21 days after the treatment. Western blot or immunofluorescence also detected the Ataxin-2 and p-TDP43 protein levels within these mice's cortex and spinal cord. Compared to PBS-treated *TDP-43*$^{Tg/Tg}$ mice, *TDP-43*$^{Tg/Tg}$ mice treated with different doses of AAV-hfCas12Max-g*ATXN2* had improved gait ($p<0.05$), decreased kyphosis ($p<0.05$), reduced tremors ($p<0.05$), and increased weight ($p<0.05$) in a dose-dependent manner (Figure1a-d), suggesting that the AAV-hfCas12Max-g*ATXN2*-treated *TDP-43*$^{Tg/Tg}$ mice improved motor coordination and neuromuscular function. Notably, high-dose AAV-hfCas12Max-g*ATXN2* treatment dramatically extended the lifespan in *TDP-43*$^{Tg/Tg}$ mice. Median survival soared by ~130 days (154.5 vs. 24 days; $p < 0.05$, with mean survival also extending by ~103 days (126 ± 25.97vs. 23 ± 0.67 days; $p < 0.01$) (Figure 1f). Remarkably, 50% (6 out of 12) of high-dose-treated mice surpassed 100 days of survival. NGS results showed that the editing efficiency of *Atxn2* in the cortex and spinal cord of *TDP-43*$^{Tg/Tg}$ mice treated with low-, middle-, and high-dose was increased with 12.73±2.01%, 22.13±3.97%, 35.60±6.93% in cortex and 3.13±0.19%, 8.50±1.86%, 9.60±1.35% in spinal cord, respectively, at 21 days after dosing. After treating *TDP-43*$^{Tg/Tg}$ mice with high-dose, the Ataxin-2 protein level in the cortex decreased to 59.37±17.32% compared to the control mice. Immunofluorescence results also showed a significant knockdown of Ataxin-2 protein level in the cortex and spinal cord p-TDP43 level in the motor cortex, almost rescued at the WT mice level. Our findings suggest AAV-mediated hfCas12Max-g*ATXN2* is an innovative approach that holds promise in extending lifespan and enhancing motor function and neuromuscular performance in ALS.

P0314

Challenging the *MECP2* gene dosage dogma by revealing new functions of this epigenetic factor

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MECP2 gene plays a critical role in neuronal cells by modulating chromatin organization and transcription control. The importance of this factor is highlighted by the fact that both its absence and overexpression can lead to severe neurological disorders as Rett syndrome (RTT) and MECP2 duplication syndrome (MSD), respectively. Notably, the pathological phenotypes of both disorders can be reversed in mice by restoring the appropriate Mecp2 dosage. Consequently, it has long been believed that a major challenge for RTT gene therapy is maintaining MeCP2 levels within a

narrow physiological range. However, surprisingly, our recent observations have indicated that an overtly excess of Mecp2 does not induce detectable behavioral changes in adult mice. Based on these findings, we hypothesize that Mecp2 overexpression has opposing effects depending on when it occurs and in which neural cell types.

To investigate the molecular root of this phenomenon, we performed a deep molecular characterization of the transcriptional and epigenetic changes induced by Mecp2 overexpression in primary mouse neural stem cells (NSCs) or mature neurons. These analyses confirmed that Mecp2 overexpression significantly altered the chromatin state and triggered substantial transcriptional changes in NSCs. Conversely, mature neurons exhibited only marginal molecular alterations. Notably, we discovered that Mecp2 aberrantly binds to developmental bivalent genes in NSCs, leading to their activation and accelerated differentiation into neurons.

In summary, our findings support recent evidence suggesting that Mecp2 can directly activate genes, thereby disrupting the normal neurogenic trajectory of precursor cells. In contrast, the overexpression in neurons, that naturally express higher levels of Mecp2, resulted in minimal effects. Thus, our data suggest that Mecp2 overexpression appears to be significantly better tolerated in post-mitotic cells compared to their progenitors. Overall, our work challenges the current prevailing view of the absolute necessity for maintaining tight MeCP2 regulation in adulthood and sheds new light on the molecular function(s) of Mecp2. These results have the potential to significantly impact the development of future RTT gene therapy approaches and refine our understanding of this crucial epigenetic factor.

P0315

AAV-mediated *DARS1* gene replacement rescues HBSL leukodystrophy in mice

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Leukodystrophies are a large group of genetic disorders that affect the white matter of the central nervous system (CNS). These diseases are typically progressive and can lead to severe neurological dysfunction, physical disability, and reduced life expectancy. The leukodystrophy Hypomyelination with Brainstem and Spinal cord involvement and Leg spasticity (HBSL) is caused by biallelic loss of function mutations in the cytosolic aspartyl-tRNA synthetase gene, *DARS1*. The HBSL disease mechanism is currently poorly understood and there are no curative therapies available.

Our group has developed a suite of adeno-associated viral vectors (AAVs) for targeted delivery of functional human *DARS1* to specific CNS cell populations. Codon optimisation of the *DARS1* coding sequence using DNA triplets with higher prevalence significantly increased levels of transgene expression in comparison to wildtype *DARS1*. Additionally, cytosine-phosphate-guanine (CpG) motifs – molecular patterns that can trigger innate immune responses – were removed from the optimised *DARS1* sequence to reduce vector immunogenicity and promote long-term transgene expression. For the initial HBSL gene therapy proof-of-concept, we selected the AAV.PHPeB capsid serotype to ensure maximum treatment effects in our C57Bl/6J mouse HBSL models.

Parallel to the vector development, we have generated HBSL disease models in mice, through conditional *Dars1* deletion in oligodendrocytes (*Dars1*^{OligoKO}) and neurons (*Dars1*^{NeuroKO}).

Both *Dars1*^{OligoKO} and *Dars1*^{NeuroKO} mice exhibit pronounced HBSL-like phenotypes, including severe motor deficits and shortened lifespan. These models facilitate critical pre-clinical proof-of-concept studies for AAV-mediated *DARS1* gene replacement and have provided new insights into the HBSL pathophysiology including identification of cell target classes for the *DARS1* gene therapy.

Combining the HBSL disease models with our optimised AAV.*DARS1* gene delivery vectors, we have demonstrated the efficacy and safety of this novel gene replacement strategy for the leukodystrophy HBSL. Systemic administration of AAV.*DARS1* vectors rescued HBSL-like disease in our severely affected HBSL models, providing proof-of-therapeutic-concept for the genetic treatment of HBSL. More broadly, these results and our targeted gene delivery and disease modelling platforms will aid development of AAV-mediated gene replacement therapies for other leukodystrophies and similar monogenic diseases of the CNS.

P0316

A novel and empowered TARGETED gene addition approach at a relevant microglia locus for the treatment of X-linked Adrenoleukodystrophy

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Adrenoleukodystrophy is an X-linked disorder (X-ALD, OMIM: 300100) resulting from a mutation in the ABCD1 gene, which causes a defect in peroxisomal beta-oxidation and leads to the accumulation of saturated very long chain fatty acids in body tissues primarily impacting the Central Nervous System (CNS). Its pediatric manifestation, named Childhood Cerebral ALD, still represents a frequent cause of unrelenting neuropathology and death early in infancy. Although allogeneic Hematopoietic Stem and Progenitors cells (HSPCs) transplantation and lentiviral gene therapy (GT) for X-ALD are considered valuable treatment options for childhood cerebral X-ALD patients, their use is limited by the slow engraftment of transplanted cell progeny in the CNS as compared to the rapid progression of neurodegeneration. Moreover, the allogeneic transplant is affected by donor availability and graft versus host disease, whereas lentiviral gene therapy by the risk of clonal expansion events consequent to vector integration.

We have identified CX3CR1, a chemokine receptor expressed on microglia that binds fractalkine (CX3CL1) and regulates recruitment to sites of neuroinflammation and microglia ontogeny, as a key locus to be targeted to enhance the ability of HSPCs to generate microglia-like cells (MLC) upon transplantation. Indeed, we demonstrated that transplantation of CX haploinsufficient HSPCs results in a faster and improved engraftment and generation of MLCs as compared to wild type cells, as well as a more efficient acquisition of mature microglia-like phenotype by the transplanted cell progeny. Based on this evidence, we have developed a CRISPR/Cas9 gene editing and targeted gene addition strategy to insert the human ABCD1 cDNA at the CX3CR1 locus to simultaneously generate a haploinsufficiency condition at the locus and drive the expression of the therapeutic cassettes under the control of the CX3CR1 locus. This could allow coupling an anticipated microglia replacement effect by the gene corrected cells with a regulated microglia-specific expression of the therapeutic gene, also reducing the safety concerns related to the use of integrating vectors.

We targeted a promoter less, splice trapping cassette encoding hABCD1 cDNA to a CX3CR1 intron achieving highly efficient targeted insertion and regulated transgene expression in microglia cell lines and human HSPCs. Our data, demonstrate a successful and efficient (up to 60% of targeted alleles) transgene integration in hHSPCs, a robust and CX3CR1_promoter regulated hABCD1expression in HSPCs progeny cells upon myeloid differentiation and the establishment of a CX3CR1 haploinsufficiency maintaining a functional copy of the gene. We are now reproducing these data employing engineered Lipid Nanoparticles (LNPs) for the delivery of gene editing reagents to CD34+ HSPCs to alleviate the well-known effects of the editing procedure and establish a new harmless ex vivo gene editing protocol with improved tolerance to cell manipulation, as well as testing protocols compatible with large scale manipulation. Simultaneously, we are testing the CX3CR1 edited human HSPCs into relevant immunodeficient models allowing to either enhance microglia engraftment (IL34 and CSF1 NSG models) or to establish proof of concept data in correcting the disease associated biochemical abnormalities (in the NSG SGM3 ABCD1-KO (Δ897) disease model). Early in vivo data will be presented.

P0317

Bridging HEK293-Produced and Sf9-Baculovirus-Produced AAV Gene Therapy in the Clinic – Evaluation of PR006 (LY3884963), an Investigational AAV9-GRN Gene Therapy for Frontotemporal Dementia Patients with GRN Mutations

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PROCLAIM is an ongoing open-label Phase I/II trial (NTCT04408625) designed to investigate the safety, tolerability, and efficacy of PR006 in patients with frontotemporal dementia caused by pathogenic heterozygous mutations in the GRN gene (FTD-GRN). PR006 is administered as a one-time injection to the cisterna magna, and participants are followed for a total of 5 years. During the study an advanced, second-generation PR006 production process was developed providing improved product characteristics and yield. A bridging cohort was initiated to evaluate the comparability of first-generation PR006, produced with a HEK293 platform (PR006 v1.0), with second-generation PR006, produced with an Sf9-baculovirus platform (PR006 v2.0).

Prior to initiating the bridging cohort, preclinical comparability studies assessed efficacy and safety endpoints in the *Grn*-KO mouse model. Evaluation of progranulin level and keypathological features of FTD-GRN recapitulated by the model in a head-to-head comparison was performed. Intracerebroventricular administration of both PR006 v1.0 and PR006 v2.0 led to a significant reduction of lipofuscin and ubiquitin accumulation, as well as a decrease in proinflammatory cytokine expression and microgliosis. Safety endpoints revealed no adverse PR006A-related histopathological findings. Based on these findings, a 6-month GLP toxicity study in cynomolgus macaques was initiated, which revealed no adverse in-life observations or histopathological findings. Based on these findings, enrollment in the bridging cohort was initiated.

We present interim biomarker analysis of 13 subjects receiving PR006 v1.0 (6 low-dose PR006 v1.0 and 7 mid-dose PR006 v1.0) and 5 subjects receiving PR006 v2.0 (3 low-dose PR006 v2.0 and 2 mid-dose PR006 v2.0). Biomarker analysis demonstrates PR006 v1.0 and v2.0 restore

cerebrospinal fluid (CSF) progranulin in a similar manner to levels at or above the normal range by month two. Biomarkers of lysosomal function were also normalized to a similar extent in participants receiving both PR006 v1.0 and PR006 v2.0.

Biomarker analysis of participants with FTD in the PROCLAIM study shows that treatment with both PR006 v1.0 and PR006 v2.0 result in similar normalization of CSF progranulin and biomarkers of lysosomal function, suggesting that PR006 may be safe and efficacious in patients with FTD-GRN and supporting further clinical development.

P0318

A homeostatic modulation of gene therapy products to treat retinitis pigmentosa

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A growing number of studies show that transcriptional modulation is emerging as an approach in the most diverse areas of gene therapy. In the case of transcriptional silencing can be achieved by cis-sequence mutagenesis of regulatory sequences by CRISPR/Cas9 or base editors (BE) or in trans with DNA binding proteins tethered to repressor domains such as epigenetic modifiers. In our efforts to develop a treatment for Rhodopsin-Autosomal Dominant Retinitis Pigmentosa (RHO-ADRP) a blinding untreatable retinal degeneration, we successfully established a robust proof of principle by using an unconventional gene silencing of endogenous RHO (Zinc Finger (ZF) DNA-binding protein (ZF-RHO) that blocks Rhodopsin (RHO) expression without the aid of tethered repressor domains). We combine this mode of RHO silencing with gene replacement (substitution with a correct copy of RHO). This approach involves the incorporation of two expression cassettes in a single AAV vector. Because engineered promoters contained in gene therapy vectors may induce an ineffective low dose or a toxic overdose expression, to de-risk our system from inefficacy and toxicity, here, we investigated whether using the gene expression regulatory syntax of RHO itself enables an “homeostatic” expression of the silencing (ZF6) and replacement (exogenous RHO) gene therapy components. We recently found a compact Rho regulatory unit (RRU) which control RHO expression by a novel gene regulatory code (Rosa Maritato et al., A DNA base-specific sequence interposed between CRX and NRL contributes to RHODOPSIN expression. bioRxiv 2023.12.22.573078; doi: https://doi.org/10.1101/2023.12.22.573078), which here we enforce a system to control silencing and replacement. By using these properties, we obtained both a fixed mean therapeutic expression levels and proper distribution of the transgenes of the silencing and replacement across transduced retina, while maintaining low AAV vector doses. Our findings provide a promising advancement in the gene therapy treatment of RHO-ADRP, ensuring effective and safe intervention for this and potentially other blinding disorders.

P0319

Generating human-evolved, cell-type-specific AAV capsids for targeted gene delivery

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Targeted delivery of therapeutic genes remains a defining challenge in gene therapy. The ideal viral delivery system would target the cell or tissue type of interest with minimal tropism for other cell types or organ systems. This would enable low dose, local delivery of a gene therapy, limiting toxicity and associated immune responses. Adeno-associated viral (AAV) vectors are safe, versatile and have emerged as the preeminent delivery vehicle for gene therapies. A major advantage of AAVs is that their tropism can be directed towards a particular cell type by recombinant engineering of the capsid through directed evolution. However, the major drawback of directed evolution is the highly species-specific properties of the new vector. As directed evolution strategies typically occur in animal models this severely hinders translatability of AAV vectors into the clinic.

Sania takes a “human-first” approach to AAV vector development to generate novel, targeted vectors that can be successfully and rapidly translated into the clinic. Our proprietary platform, R-Scan, utilises a combination of human induced pluripotent stem cell (IPSC) derived cell types and microfluidics to perform directed evolution in an in vitro system. Using R-Scan, we can generate capsids for targeted delivery of genes to several neural circuits to treat a broad range of disorders.

Our initial clinical target is spasticity, a prevalent disorder characterised by painful and uncontrollable muscle contractions that currently has poor treatment options. The underlying cause of spasticity is motor neuron hyperexcitability. We used R-Scan to evolve AAV vectors that target motor neurons directly via intramuscular injection by recreating this spinal-motor neural circuit using a combination of IPSC-derived motor neurons and myocytes. To ensure we capture the phenotypic diversity of the human population we use a pooled line of IPSCs from multiple donors. For capsid screening Sania generated 16 different proprietary capsid libraries based on six parental serotypes and three diversification strategies – 1) random mutagenesis, 2) peptide display of random peptide sequences and, 3) peptide display of rationally designed motifs known to target our cell type of interest.

So far, R-Scan has generated multiple AAV vectors that show superior transduction of both human motor neurons in vitro and human neuromuscular organoids. These capsids also show efficient transduction of mouse motor neurons following intramuscular injection, demonstrating translatability from our in vitro system to whole animal. Biodistribution analysis shows that intramuscular injection of these AAVs prevents vector spread and transduction in off target tissues. Additionally, we have shown that our novel capsids can be manufactured to similar titers as wild type vectors.

We are further developing R-Scan so we can generate human-evolved vectors for multiple neuronal circuits and cell types. Ultimately, our goal is to utilise R-Scan to deliver targeted, safe, and enduring AAV gene therapies for disorders of high unmet need.

P0320

Fine-tuning of therapeutic gene regulation is essential for functional improvement in FAM161A-deficient ciliopathy

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Gene replacement is the most intuitive approach to recessive diseases, and has spearheaded the development of gene therapy. Defects in the *FAM161A* gene encoding for **two isoforms** of a ciliary protein have been proven to cause autosomal recessive retinitis pigmentosa (RP28). FAM161A is a protein expressed in the basal body and in the inner scaffold of the connecting cilium of photoreceptors. Upon FAM161A loss, the cilium structure disorganizes and the outersegments progressively collapse (Mercey *et al.* 2022). This study aims to determine the efficiency of these isoforms to restore the connecting cilia and improve retinal function after gene transfer. We designed and produced AAV2/8 vectors expressing the human isoforms HL (with exon 4) or HS (without exon 4) under a composite IRBP-GRK1 promoter or promoters derived from the human *FAM161A* regulatory regions FCBR1-F0.4 or FCBR2-F0.4. We injected these vectors at 2 weeks of age in *Fam161a*$^{tmb/tmb}$ mice, shortly after the start of cilium disorganization, and evaluate FAM161A expression, retinal function and structure up to 3 months post-injection. While IRBP-GRK and FCBR2-F0.4 promoters lead to FAM161A expression not only in the connecting cilium but also in the inner segment and photoreceptor cell body, FCBR1-F0.4 faithfully reproduces WT exclusive connecting cilium expression and reassemble the inner scaffold of connecting cilium as assessed by Ultrastructure expansion microscopy (U-ExM). Both IRBP-GRK1 and FCBR1-F0.4 vectors preserved retina thickness and outersegment structure. For IRBP-GRK vectors, only combined administration of HL and HS expressing vectors maintain an outer nuclear thickness similar to wild type condition, but not retinal function. Finally, only the combined administration and high doses of FCBR1-F0.4 HL and HS vectors is able to correctly reconstruct the cilium structure (evidenced by U-ExM), preserve the same number of connecting cilia as wild type retina and to improve retinal function up to 3 months post-injection. Our work demonstrates that fine-tuning of the therapeutic gene regulation is essential for retinal function restoration. Not only the promoter, but also the dose and the isoforms are conditions which should be carefully addressed to securely propose gene therapy for diseases linked to structural proteins such as FAM161A.

P0321

Gene Therapy enhancing hPD-L1 expression in Hematopoietic Stem Cells: an innovative approach for Multiple Sclerosis Treatment in the disease animal model

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Multiple sclerosis (MS) is a debilitating chronic neurodegenerative disease characterized by an abnormal immune response mediated by auto-reactive activated T cells leading to inflammatory demyelination in the central nervous system (CNS). MS has an unmet medical need. Transplantation of autologous hematopoietic stem and progenitor cells (HSPCs) has reported some benefit in selected MS patients, but its use is still controversial. The limited success of this and other immuno-modulatory treatments is likely due to the complex pathogenesis of MS, which involves the loss of immune tolerance and is influenced by a combination of various factors. Among them, the dysregulation of programmed death-ligand 1 (PD-L1) / PD-1 axis that reasonably plays a central role. Recent studies have shown a significantly reduced expression of PD-L1 in HSPCs of MS patients and murine models, similarly to what reported in other autoimmune diseases, further supporting its critical involvement in the disease process.

Based on these findings we designed an innovative lentiviral-based HSPC gene therapy approach for MS aimed at enhancing HSPC immune-regulatory properties by genetic engineering for hPD-L1 expression. The ability of PD-L1 overexpressing HSPCs to mitigate the clinical course of the disease was investigated in the experimental autoimmune encephalitis (EAE) MS animal model, employing an innovative, CNS-targeted cell delivery approach based on intrathecal lumbar injection (ITL), in the absence of any conditioning regimen. Transplantation of hPD-L1 over-expressing HSPCs in these experimental conditions resulted in a significant benefit in the treated EAE mice, with mitigation of the severity of the disease and rapid recovery from the symptoms. This positive effect was particularly pronounced with the ITL cell transplantation, which proved to be more effective in limiting the progression of EAE disease than standard intravenous cell delivery. Consistently, PD-L1 HSPC transplantation resulted in a reduction of the overall CNS inflammation and of the demyelination load in the CNS. A cell biodistribution study conducted short term after transplant showed that these long-term effects follow a short-term homing of the transduced HSPCs into disease target organs where they are clearly detectable up to at least one week post-transplant. The actual role of the cell homing process in positively affecting the CNS environment has been investigated by a single-cell RNA analysis conducted on sorted PD-L1 expressing HSPCs collected at the peak of their frequency in the CNS of the transplanted mice.

Overall, these data provide the evidence that PD-L1 HSPC gene therapy may represent a valuable therapeutic option for MS, possibly enhancing the immune-modulatory role exerted by autologous HSPCs, thus paving the way for the development of the approach towards clinical testing.

P0322

A new intravenous gene therapy to treat neurodegeneration in a mouse model of Pelizaeus Merzbacher disease

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Pelizaeus Merzbacher disease (PMD) is the most frequent inherited defect in myelin formation in the central nervous system (CNS). This X-linked hypomyelinating leukodystrophy is due to pathogenic variants in the proteolipoprotein gene (PLP1). This gene encodes two major CNS myelin proteins, PLP and its spliced isoform DM20, which play a crucial role in the ontogenesis of myelinating cells (oligodendrocytes) and in the compaction and maintenance of the myelin sheath. PMD is characterized in male patients by a myelination disorder similar to that seen in premature infants, followed by a progressive length-dependent axonal damage which causes the severity of the disease and leads to death between the second and fourth decades of life. This neurodegeneration has been shown to be linked to mitochondrial damage.

Currently, there is no effective preventive or curative treatment to treat this severe axonal degeneration observed in PMD patients.

Our aim was to develop an intravenous gene therapy based on Intravenous AAV delivery encoding a confidential target (patent ongoing – embargo until September 2024), to target mitochondrial damage in neurons and thereby treat neurodegeneration in PMD. To test our strategy, we chose the Plp-Tg66/66 mouse model, which reproduces the symptomatology and pathophysiology of the most common form of PMD caused by duplication of the PLP1 gene. After a retroorbital intravenous injection at 3 weeks of age, we assessed weekly weight follow-up and evaluated motor skills at 1 month and 2 months of age. We performed euthanasia at 2 months after the last behavioral assessment to collect CNS and peripheral tissues for molecular, histological, and biochemical analysis. We compared all our data with non-treated Plp-Tg66/66 mouse and non-affected mouse controls.

Results demonstrated a beneficial therapeutic effect on weight, motor behavior and neuroinflammation in our treated mouse model in comparison to non-treated affected animals. All data will be presented.

P0323

Cell-based device provides effective therapeutic strategy to treat the metachromatic leukodystrophy

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Metachromatic leukodystrophy (MLD) is a lysosomal storage disorder caused by an inherited deficiency of arylsulfatase A (ARSA) and characterized by accumulation of sulfatides in both glial cells and neurons leading to myelin degeneration in the central and peripheral nervous systems. Currently, Libmeldy©, an Ex vivo gene therapy, is the standard of care for pre-symptomatic MLD patients, while no effective treatment is available for patients once symptoms have appeared. Here, we investigated an innovative approach to deliver recombinant ARSA by encapsulated-cell therapy which maybe a therapeutic option for symptomatic patients. A cell-based device is capable of continuously delivering human ARSA without the need for weekly intrathecal injections of rARSA, an approach which is under clinical evaluation. Two constructs of genetically engineered cells have been tested to determine which one is targeting the central nervous system more efficiently. Consequently, a dose response evaluation has been performed with 2 doses (cells number loaded) to evaluate the best therapeutic effect. MLD mice were implanted at 6 months of age once disease is well established. Device biocompatibility as well as therapeutic efficacy were evaluated 3 months after implantation. The device was well tolerated in animals and we were able to demonstrate a correction of sulfatide storage and significant improvement of neuroinflammation in all treated mice.

Next, Myo-P device encapsulating ARSA expressing cells have been successfully implanted subdurally in 2 NHP and safety and efficacy data will be provided after 6 months implantation.

All these data support the rationale of using Myo-P encapsulated cell therapy in symptomatic MLD patients.

P0324

Liver-directed gene therapy with chimeric GALC enzyme provides widespread enzymatic correction and survival advantage in a murine model of globoid cell leukodystrophy

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Globoid Cell Leukodystrophy (GLD) is a rare genetic neurodegenerative lysosomal storage disease (LSD) caused by deficient b-galactosylceramidase (GALC) enzyme activity. To date, there is no cure for GLD. Various pharmacological, cell, and gene therapy (GT) strategies leverage the cross-correction mechanism, where secreted lysosomal enzymes are recaptured by GALC-deficient cells, showing benefits in pre-clinical models. However, achieving widespread enzymatic reconstitution and pathology correction in all the affected tissues, especially the central and peripheral nervous systems (CNS, PNS), remains challenging. Pre-clinical and clinical studies indicate a correlation between enzyme availability, clearance of intracellular storage, and therapeutic benefit. Chimeric lysosomal enzymes with increased secretion and blood-brain barrier (BBB) penetration have shown improved efficacy of GT approaches in murine models of

neurodegenerative LSDs. Therefore, developing chimeric GALC enzymes with increased bioavailability could enhance GT approaches for GLD.

In vivo liver-directed GT has successfully alleviated inherited coagulation disorders with a single intravenous administration of lentiviral vectors (LVs) or adeno-associated viral (AAV) vectors expressing functional transgenes from hepatocytes. We hypothesized that liver-directed GT using LVs expressing GALC enzymes with enhanced bioavailability could provide a minimally invasive, one-time treatment for GLD, ensuring a stable GALC supply to tissues via the bloodstream.

To this end, we generated LVs carrying chimeric GALC construct designed for improved secretion and BBB penetration using a hepatocyte-specific expression cassette. We evaluated the safety and efficacy of this construct *in vitro* in a human hepatocyte cell line and neural stem cells (NSCs) derived from a GLD mouse model. LV-transduced hepatocytes exhibited supraphysiological GALC activity, with chimeric construct outperforming the native GALC in enzyme production and secretion. The secreted chimeric enzyme was effectively recaptured by GLD neural cells, clearing intracellular galactocerebroside (GalCer) storage.

Building on these *in vitro* findings, we administered LVs expressing chimeric or native GALC to newborn GLD mice for *in vivo* testing. The copies of integrated LV in the liver of treated mice correlated with enzyme activity, reaching supraphysiological levels (70X). Elevated enzyme activity in the sera of treated mice corresponded with restored GALC activity in the spleen, CNS, and PNS tissues, demonstrating that GALC circulates in the bloodstream and cross-corrects distant tissues. Enhanced tissue bioavailability was associated with increased survival in treated mice.

These findings support the feasibility and safety of liver-directed GT for GLD using chimeric GALC transgene.

P0325

PacMan: A novel gene editing strategy to remove disease-causing repetitive sequences

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More than forty diseases, most of which affect the nervous system, are believed to be caused by expansions of short DNA sequence repeats in specific genes. Generally, the larger the expansion, the more severe the disease progression.

In this study, we propose a novel CRISPR-based gene editing approach, PacMan, designed to reduce the number of disease-causing DNA repeats. PacMan leverages MMEJ-assisted DNA repair to delete the repetitive sequence. Specifically, CRISPR is employed to induce a double-strand break at a specific position, utilizing sequence homology around the cleavage site to decrease the number of DNA repeats through repetitive cleavage and repair though deletion at the target site.

To test the PacMan strategy, we designed an approach for Huntington's Disease (HD), a neurodegenerative disorder caused by CAG repeat expansions in the HTT gene. We evaluated the efficacy of PacMan in various cell lines and in vitro HD cellular models, demonstrating that PacMan can reduce the number of CAG repeats with up to 6% efficiency in HD patient IPSC-derived post-mitotic neurons. The majority of reduction events involved decreasing the repeats from a range of 20 to 43 CAG repeats down to 2 CAG repeats. Additionally, inhibiting DNAPK with small molecules and thereby skewing DNA repair from NHEJ to MMEJ, increased the efficacy of CAG reduction by 2 to 3-fold while decreasing unintended outcomes.
Our ongoing efforts focus on enhancing PacMan's efficiency through modulation of DNA repair pathways and the application of novel Cas9 enzymes. In summary, we present a novel editing strategy to reduce disease-causing repetitive sequences. With further refinements, PacMan has the potential to ultimately provide a cure for repeat expansion disorders.

P0326

CRISPRa-engineered human neural stem cell therapeutics to overcome remyelination failure in Multiple Sclerosis

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Neural stem cell (NSC) transplantation is promising for central nervous system (CNS) repair by facilitating cell replacement, providing trophic and immunomodulatory support, and promoting remyelination. This approach has recently entered clinical trials for multiple sclerosis (MS). However, challenges such as large-scale production and the need for immunosuppressive regimens limit its clinical application, highlighting the potential of alternative cell sources like pluripotent stem cells. Additionally, the complex dysregulation of the physiological environment in MS may inhibit the recruitment, regeneration, and remyelination capabilities of transplanted NSCs.

Engineering NSCs to overexpress anti-inflammatory and neurotrophic molecules has shown improved recovery in experimental MS models, supporting the optimization of NSCs to address remyelination failure in MS. We hypothesize that targeted and inducible expression of key oligodendroglial and anti-inflammatory/trophic genes (therapeutic genes, TGs) in hiPSC-derived NSCs using CRISPRa-based editing will enhance their differentiation into pure, homogenous OPC/OL populations with increased therapeutic plasticity. This should improve their safety and efficacy for cell replacement and remyelination by overcoming the non-permissive barriers of MS.

We designed and tested multiple gRNAs targeting TGs to achieve consistent up-regulation upon transient delivery with CRISPRa in hiPSC-derived NSCs. To identify transcription factors (TFs) that enhance OPC yield, we engineered hiPSCs with Lentiviral Vectors (LVs) encoding inducible CRISPRa and selected gRNAs targeting different combinations of OPC TFs. During NSC differentiation, we achieved inducible, robust, time-dependent overexpression of OPC TFs. Combining TF up-regulation with an established differentiation protocol, we observed increased expression of OPC/OL markers by qRT-PCR and FACS analysis, indicating enhanced OPC yield and purity after 14 days of differentiation.

We are evaluating the effects of TG expression on NSC biology, multipotency, and safety compared to untreated matched cultures using classical and genome-wide approaches. The anti-inflammatory activity of TG-expressing NSC will be assessed through co-culture systems with stimulated PBMCs or T cells, examining allogeneic immune responses and cytokine profiles. Neurotrophic properties and OPC differentiation capability will be evaluated in vitro under basal conditions and an “MS-like environment” focusing on neuronal and OPC differentiation/survival compared to untreated and non-induced NSC.

This study aims to advance novel therapeutic approaches based on enhanced and personalized cell products to address unmet clinical needs in MS, particularly remyelination failure.

P0327

Gene Editing with a Type V CRISPR-Cas Enzyme Disrupting the Aberrant Splicing of STMN2 as a Potential Treatment for ALS

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TDP-43 is a nuclear RNA-binding protein that regulates RNA processing. In many neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTD), TDP-43 is mis-localized and accumulated in the cytoplasm, a pathological hallmark of these conditions. The lack of nuclear TDP-43 in disease contexts results in aberrant splicing and processing of numerous genes. One of the most highly down-regulated gene is STMN2, which encodes for a microtubule-binding protein that controls axonal outgrowth and is highly expressed in motor neurons. Prior studies suggest that nuclear depletion of TDP-43 results in the aberrant splicing of STMN2 and loss of full-length functional STMN2, which may contribute to the pathology in ALS. To explore a potential treatment for ALS, we used gene editing with a Type V CRISPR-Cas enzyme to disrupt aberrant splicing of STMN2 by creating large deletions of ~10 base pairs.

We first screened in HEK cells multiple type V CRISPR nucleases and guides spanning the 310 base pair aberrantly spliced novel exon of STMN2 (exon 2A) that includes cis-acting elements such as a splice acceptor site, a TDP-43 binding region and a polyadenylation site. Next, nuclease/guide pairs with efficient indel formations were tested in SH-SY5Y cells under TDP-43 knockdown to identify the pairs that could reduce aberrant splicing and promote an increase of full-length STMN2 mRNA. Several nuclease/guide pairs disrupting the splice acceptor site resulted in the effective reversal of aberrant splicing and increase of full-length STMN2 mRNA, but not the other cis-acting elements, thus defining the splice acceptor as the relevant target. These findings were replicated in human induced pluripotent stem cell (iPSC)-derived motor neurons where no potential off-targets were confirmed while the on-target activity increased over time. Moreover, the disruption of aberrant splicing using the nuclease/guide pairs in human motor neurons reversed the axonal loss caused by TDP-43 knockdown. Finally, nuclease/guide pair ABR-001 variant and gX, and ABR-004 variant and gY, were packaged into a rh10 AAV vector serotype and injected into neonatal mice transgenic for the human STMN2 exon 2A

locus. In these mice, efficient editing as well as the reversal of STMN2 aberrant splicing were observed.

In summary, we have identified multiple type V nuclease/guide pairs that reverse the aberrant splicing of STMN2 and the disease-relevant phenotypic consequences due to TDP-43 nuclear depletion in a cell line, in motor neurons, and in a mouse model. Together, these findings have the potential to pave a path towards a CRISPR gene editing therapeutic for ALS.

P0328

CRISPR/hfCas13Y or hfCas12Max as gene-editing therapy for Angelman syndrome

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Angelman syndrome (AS) is a rare, severe neurodevelopmental disorder often diagnosed in early childhood that primarily affects the central nervous system. Intellectual disability, sleep dysfunction, seizure disorders, impaired cognition and speech, motor deficits, and microcephaly characterize Angelman syndrome. Although AS patients have a normal life expectancy, they require lifelong care with no disease-modifying treatment options. Because paternally-inherited ubiquitin-protein ligase E3A (*UBE3A*) is epigenetically silenced in *cis* by a long non-coding antisense transcript (*UBE3A-ATS*) in neurons, mutation or deletion of maternal *UBE3A* mediates the targeted degradation of several protein substrates, resulting in near complete loss of *UBE3A* in the brain. However, the intact paternal *UBE3A* can be unsilenced with gene-editing nucleases by prematurely truncating *Ube3a-ATS*. In our previous study, adeno-associated virus (AAV)-mediated high-fidelity (hf) Cas13X has been shown to restore the expression of paternal *Ube3a* in a mouse model of AS. The treated mice improved body weight and motor function, but not in the marble burying test or brain weight. Here, we optimized our gene-editing tools for the treatment of AS by enhancing the editing efficiency of AAV vector packaged high-fidelity Cas13Y (hfCas13Y) or Cas12Max (hfCas12Max) to facilitate the expression of Ube3a. We designed and constructed a series of AAV transfer plasmids with modified promoters, engineered gRNA, and added *cis*-regulatory motifs. These optimized AAV vectors were then intracerebroventricularly administered to the AS mice on postnatal day 1 to evaluate the editing efficiency of *Ube3a-ATS* silencing *in vivo*. The RNA and protein of reinstated Ube3a in the dissected brain regions were measured by RT-qPCR or western blot (WB). Our results demonstrate the effectiveness of the optimized AAV-hfCas13Y and AAV-hfCas12Max systems. When delivering four versions of optimized AAV-hfCas13Y (V1, V2, V3, and V4) into the neonatal AS mice, we observed the paternal Ube3a protein expression in the cortex and hippocampus of AS mice surpassed 50% of that found in wild-type (WT) mice at 4 weeks post-infection with AAV-hfCas13Y V4. The AAV-hfCas13Y V4 dramatically reduced the RNA level of *Ube3a-ATS* by 50%. Similarly to the CRISPR/hfCas13 RNA-editing findings, the AAV-hfCas12Max system also showed promising results. AAV-hfCas12Max V2-treated AS mice exhibited the highest Ube3a expression in the cortex and hippocampus, similar to that in WT mice. WB results showed that AAV-hfCas12Max V2 significantly increased the UBE3A expression. These optimized AAV vectors, which encapsulated both the hfCas13Y and the hfCas12Max systems, could significantly enhance the expression of UBE3A, with AAV-hfCas12Max showing particularly robust results. Our findings support the continued development of CRISPR/hfCas12Max gene-editing therapy, and additional studies are ongoing, including body weight and behavior tests. The knockdown

of *Ube3a-ATS* RNA mediated by AAV-hfCas13Y or hfCas12Max may represent a more favorable approach for AS treatment. Overall, our study demonstrates a novel potential gene-editing therapy for Angelman syndrome.

P0329

Wnt-induced axon guidance of photoreceptors

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Photoreceptors are the sensory neurons of the eye detecting light. In retinal degenerations the loss of these photoreceptors is critical as they are unable to regenerate. Transplantation studies have shown the successful survival of stem cell derived photoreceptors in murine models of advanced retinal degeneration.

For photoreceptor replacement therapy to be effective, donor photoreceptors must form new synaptic connections with the host retina with high efficiency. Studies currently show qualitative data on photoreceptors forming limited synaptic connections between the donor transplanted cells and the host bipolar cells. A recent report identified a role for Wnt5a in promoting rod/bipolar synapse formation in retinal development. Here, we sought to explore this further with the aim of re-introducing it into the host retina to promote donor cell neurite outgrowth and synaptic connectivity in transplantation.

For the assessment of Wnt-induced neurite growth extension, on murine rod photoreceptors an *in vitro* assay was established. Upon the application of Wnt5a/b there was a significant increase in both the number of photoreceptors forming neurites, (86.4%±5.6) compared to BSA-control (34.4%±17.1; $P < 0.001$) and in the neurite length of rods, increasing to (33.7mm±15.1) compared to the BSA-control condition (19.1mm±9.5; $P < 0.001$). These results show Wnt5a/b induces both neurite formation and extension in rod photoreceptors.

To assess polarisation of photoreceptors, an in vitro microfluidics system was designed to ascertain if Wnts acted as chemo-attractants or repellents towards photoreceptors. Wnt5a induced both neurite extension and polarisation in murine rod photoreceptors towards the Wnt-treated chamber. To provide a model co-culture system to explore the pathways regulating neurite outgrowth and photoreceptor-bipolar cell synapse formation more broadly, we have identified a novel method to isolate and culture bipolar cells using CD15, a cell surface marker, resulting in a highly enriched bipolar cell population, together with Müller glia cells, which act as a cell feeder layer. Murine rod photoreceptors and bipolar cells have been co-cultured in these microfluidic devices and show some examples of axo-somatic and axo-dendritic connectivity. Further development of the microfluidic devices is required to assess the connectivity of these retinal cells in an in vitro environment.

A Wnt5a expression vector was generated and successfully mediated expression of Wnt5a in adult bipolar cells. This overexpression was combined with rod transplantation. Rod transplants with Wnt5a showed a statistically significant proportion of rod photoreceptors polarised and extending their neurites towards the host bipolar cells.

Future work will investigate if Wnts act in a similar way on cone photoreceptors and if this can be co-expression with stem cell derived human cone transplants to improve synaptic connectivity between the donor cones and the host bipolar cells.

P0330

Intra-cisterna magna administration of allele-specific AAV-based therapy alleviates neuropathology and motor deficits in Spinocerebellar Ataxia Type 3

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Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease, is an autosomal dominantly inherited and fatal neurodegenerative disorder. It is characterized by an expansion of the trinucleotide CAG repeat located in the coding region of the *ATXN3* gene. This abnormal expansion induces a toxic gain-of-function in the ATXN3 protein, leading to neurodegeneration, particularly in the cerebellum. Despite ongoing investigations, no treatment currently exists to modify disease progression in SCA3 patients. In the last years, population genetic studies have identified single nucleotide polymorphisms (SNPs) that co-segregate with the expanded CAG repeats in 70% of SCA3 patients. Based on that, in our study, we investigated the therapeutic potential of adeno-associated virus (AAV) vectors encoding artificial microRNAs designed to target SNPs present in the mutant ATXN3 mRNA (mirATAX treatment).

First, the silencing capacity and selectivity of different silencing constructs against the mutant ATXN3 mRNA were evaluated in HEK293T cell lines transiently expressing human mutant or wild-type ATXN3. Our *in vitro* results identified a lead construct that demonstrated significant and selective silencing of the mutant ATXN3 allele. Next, AAV encoding the lead silencing construct was delivered via intra-cisterna magna (ICM) single administration in a severely impaired transgenic mouse model of SCA3. Significant and robust improvements in motor behaviour were observed at 5-, 8-, and 11-weeks post-injection, assessed by rotarod performance, beam-walking testing, and catwalk XT analysis. These findings were supported by a dose-dependent reduction in the levels of mutant ATXN3 mRNA and rescue of glial markers typically dysregulated in the cerebellum of transgenic mice. Moreover, a significant increase in the levels of neuronal markers (e.g., NeuN) was observed. Histological analysis indicated a reduction in the number of ATXN3 aggregates and a trend toward preventing layer thickness shrinkage within the cerebellar lobules in treated mice. Notably, post-mortem analysis at 13 weeks post-ICM injection revealed widespread detection of AAV genome copies in disease-relevant brain regions, which strongly correlated with mirATXN3 expression. Additionally, statistically significant correlation between mutant ATXN3 knockdown levels and behaviour performance was observed. RNA sequencing was also conducted on the cerebellum of mice. Principal component analysis (PCA) revealed that the transcriptomic profile more closely resembles that of wild-type animals when compared to non-treated transgenic animals.

In conclusion, our results demonstrate widespread biodistribution, robust and durable efficiency of mirATAX treatment in the rodent brain following single vector administration. Overall, this supports allele-specific AAV-based therapy in SCA3, representing a step toward closer clinical translation for SCA3 patients.

P0331

Delivery of *APOE* protective variants for *APOE4* Alzheimer's disease: comparison of AAV capsid serotypes and route of administration in cynomolgus monkeys

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Polymorphism in the apolipoprotein E (*APOE*) gene is a major genetic risk determinant for late-onset Alzheimer's disease (AD), with the *APOE*ε4* allele conferring an increased risk and the APOE*ε2 allele conferring decreased risk relative to the common *APOE*ε3* allele. The objective of this study is to compare different adeno-associated virus (AAV) serotypes and routes of administration for optimal delivery of a protective variant of *APOE* in cynomolgus monkeys (*Macaca fascicularis*). Two AAV serotypes were compared: AAV5 and AAV9, as well as two routes of administration for cerebrospinal fluid (CSF) delivery: intracerebroventricular (ICV) and intra cisterna magna (ICM).

AAV vector DNA and *APOE* mRNA levels in the brain tissue of monkeys were evaluated using specific qPCR and RT-qPCR assays, respectively. Additionally, we measured human ApoE protein in CSF, brain tissue and plasma, using a specific ELISA, as well as NFL and GFAP as markers of neuronal damage and gliosis, respectively, using the Quanterix platform.

After a single administration of AAVs directly to the CNS, at equivalent doses between conditions, we successfully expressed human *APOE* in cynomolgus monkeys. We detected vector DNA copies and APOE mRNA molecules as well as human ApoE protein in different brain areas and CSF. No overt neuronal damage or gliosis was observed based on NFL and GFAP transient responses in CSF. The highest levels of human ApoE protein were detected after AAV9 ICV delivery of the therapeutic transgene.

Our study indicates the feasibility of ICV delivery for CNS-specific expression of a protective variant of human APOE in cynomolgus monkeys implicating a one-time administered gene therapy by overexpressing a protective APOE variant as a potential treatment for AD.

P0332

Therapeutic CRISPR/hfCas12Max-based gene-editing therapy reduces the mutant huntingtin proteins and enhances motor functions for Huntington's disease

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A potential once-and-done therapeutic therapy for the central nervous system remains a significant challenge in the treatment of neurodegenerative disorders. Huntington's disease (HD), a lethal and inherited disease, is the most frequent autosomal dominant neurodegenerative disorder with no curable treatment available. HD is caused by the CAG trinucleotide repeats in exon 1 of the huntingtin (*HTT*) gene, resulting in the production of toxic mutant huntingtin (mHtt) protein, leading to progressive disruption of neuronal physiology. These toxic gain-of-function causes progressive motor, cognitive, and neuropsychiatric impairments that appear during mid-life and slowly progressive decline over two decades until death. Partial suppression of the production of mHtt protein in the brain has been demonstrated to be both safe and efficacious in animal models of HD, providing proof-of-concept for an *HTT*-lowering therapy strategy. Here, we designed a novel CRISPR-DNA gene-editing therapy using high-fidelity Cas12Max (hfCas12Max), an engineered Cas12i variant demonstrating high editing efficiency and specificity, with guide RNA targeting HTT (g*HTT*) to reduce the mHtt protein levels. We constructed a single recombinant adeno-associated viral (AAV) vector packaging for hfCas12Max and g*HTT* (AAV-hfCas12Max-g*HTT*). Bilateral and intrastriatal injections of either AAV-hfCas12Max-g*HTT* or PBS to the striatum were performed in 3-month-old zQ175 mice, a knock-in (KI) mouse model of HD. We evaluated the indel efficiency and mHtt protein levels in the striatum 4 weeks after the injection. We then checked for mHtt protein aggregation in the striatum four months after the injection. We monitored any changes in the motor function of these zQ175 KI mice monthly beginning one month after the injection. The AAV-hfCas12Max-g*HTT*-treated zQ175 KI mice show the indel ratio of *HTT* reaching 67.5% ($p<0.001$) and the knockdown efficacy of mHtt protein of 72% ($p=0.0018$) in the striatum 4 weeks after the injection when compared to the PBS-treated zQ175 KI mice (Figure 1a). The mHtt protein aggregates in the striatum of zQ175 KI mice 4 months after injection of the AAV-hfCas12Max-g*HTT* decreased by 80% compared to those of PBS-treated zQ175 KI mice. Notably, the levels were similar to those observed in wild-type mice($p<0.01$, Figure 1b). Furthermore, AAV-hfCas12Max-g*HTT*-treated zQ175 KI mice showed shorter traverse times on the balance beam at 1-, 2-, 3-, and 4-month post-injection, suggesting the motor functions have been significantly improved (at 4 months; $p=0.0003$) when compared to the PBS-treated zQ175 KI mice at 4-month post-injection. The agility of the AAV-hfCas12Max-g*HTT*-treated zQ175 KI mice also approached that of wild-type mice, suggesting near-normalization of motor function in the beam test (Figure 1c). Our research findings not only provide a robust basis for the use of AAV-hfCas12Max-g*HTT* gene-editing therapy for the treatment of Huntington’s disease but also underscore its potential to significantly reduce the mHtt protein level and enhance motor function in a mouse model of HD. This significant breakthrough could pave the way for a new era in HD therapy.

P0333

Neuroligin 2 gene delivery into the brain alleviates seizures in rodent models of epilepsy

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Epilepsy, characterized by recurrent unprovoked seizures, affects millions worldwide and presents a considerable burden due to its associated morbidity, mortality, and diminished quality of life. Despite numerous advancements in drug therapies, approximately one-third of patients with epilepsy remain resistant to current pharmacological treatments. Gene therapy offers a novel

approach by targeting the underlying pathological processes of epilepsy directly at the cellular level. We hypothesized that gene delivery of neuroligin 2 (NL2), a postsynaptic cell adhesion molecule that enhances inhibitory synaptic function, could attenuate seizures in epileptic rodent models. First, we systemically administered an AAV.GTX (tyrosine-mutant AAV9) vector expressing NL2 under the control of the synapsin I promoter (AAV.GTX-NL2) to epileptic (EL) mice, a model of polygenic generalized epilepsy. Seizure duration, intensity, and frequency were significantly reduced in AAV.GTX-NL2-treated mice compared to control groups, with the therapeutic effects persisting for at least 14 weeks post-injection. Immunohistochemical analysis revealed widespread expression of exogenous NL2 in the brain, co-localizing with the postsynaptic inhibitory marker gephyrin. No behavioral abnormalities were observed in the open field test. To further explore the potential of NL2 gene therapy in focal epilepsy, we used a kainic acid-induced rat model of temporal lobe epilepsy. Prior to gene therapy, these rats were monitored for several months, during which time numerous daytime spontaneous seizures were observed. To achieve local NL2 expression, AAV2-NL2, which is based on an AAV2 vector, was then injected bilaterally into the hippocampi of these rats. The evaluation of seizure severity following the treatment suggested the potential therapeutic efficacy of NL2 gene therapy in this focal epilepsy model. These findings provide insights into the potential utility of NL2 gene therapy as a treatment approach for both focal and generalized epilepsy.

P0334

Investigating the effects of constitutive or late restoration of $Na_v1.1$ in GABAergic interneurons in a mouse model of Dravet Syndrome

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Dravet Syndrome (DS) is a developmental and epileptic encephalopathy characterized by drug resistant tonic-clonic seizures, psychomotor delay, cognitive impairment, and a high risk of mortality by sudden unexpected death (SUDEP). It results from haploinsufficiency of the *SCN1A* gene, encoding the alpha subunit of the voltage-gated sodium channel Nav1.1, predominantly expressed in GABAergic interneurons (IN). DS is triggered by IN hypoexcitability, but secondary circuit modifications contribute to disease progression. Significant efforts in gene therapy aim to provide a cure, with most of the strategies directed atrestoring *SCN1A* gene expression specifically in INs. We provided evidence that ubiquitous restoration of physiological levels of *Scn1a* after symptom onset is sufficient to revert seizures and behavioral alterations in a mouse model of the disease. However, since information on the optimal neuronal populations to target at different disease stages is lacking, this study investigates whether correction of GABAergic INs alone is sufficient to treat DS at various stages.

To this aim, we took advantage of a DS reversible mouse model carrying a conditionally removable STOP cassette in *Scn1a* locus (*Scn1a*$^{Stop/+}$) crossed with the constitutive or inducible GABAergic Cre drivers (Gad2-Cre and Gad2-Cre ERT2) to restore *Scn1a* gene expression in INs.

Phenotypic evaluations of *Scn1a*$^{Stop/+}$;Gad2Cre mice were conducted through survival monitoring until post-natal day P60, video-EEG recordings to detect spontaneous seizures, thermal induction to assess seizure susceptibility and whole-cell patch-clamp recordings to evaluate neuronal excitability and synaptic transmission in hippocampal circuit. *Scn1a*$^{Stop/+}$;Gad2Cre mice were fully

protected from SUDEP and febrile seizures and exhibit a substantial reduction in spontaneous seizure frequency and severity compared to *Scn1a*$^{Stop/+}$mice. Intriguingly, fast-spiking IN firing properties and inhibitory postsynaptic currents in Scn1a$^{Stop/+}$;Gad2Cre mice were not completely normalized, suggesting the existence of non-cell autonomous defects that cannot be corrected by a specific therapeutic intervention in INs.

To mimic the effect of an IN specific gene therapy administered in the chronic phase of the disease, we restored *Scn1a* gene expression in INs of Scn1a$^{Stop/+}$; Gad2CreERT2 adult mice. After seizure baseline video-EEG recordings from P45 to P60, mice were randomized and injected with vehicle or tamoxifen, that activates Cre recombinase and restores physiological Nav1.1 expression in INs. Mice are being recorded for 30 days after Nav1.1 re-expression and the effect on seizure frequency and severity will be evaluated.

This study will enhance our understanding of the impact of GABAergic IN dysfunction at different stages of DS and will inform ongoing gene therapy efforts regarding the sufficiency of correcting only GABAergic INs at various phases of the disease.

P0335

Supplementation of β-catenin in CTNNB1 syndrome using antisense-oligonucleotides

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CTNNB1 syndrome is caused by the *de novo*, loss of function mutation in one allele of the haplo-insufficient CTNNB1 gene that encodes β-catenin. β-catenin acts as a transcription factor in the canonical Wnt signalling pathway as well as supports cellular adherens junctions through interaction with various cadherins, both of which is critically important during early embryonic development. Patients with CTNNB1 syndrome suffer from a range of motor, cognitive and behavioral impairments including spasticity, hypotonia, eye and vision problems and congenital heart defects. There is currently no treatment available to ameliorate the progression of this disease. The mutations in the patients vary in type (nonsense, missense, frame-shift and splicing mutations) and do not appear to be localized to a certain part of the gene. We explored possibilities for utilization of antisense oligonucleotides (ASO), short, single-stranded and chemically modified oligonucleotides that can specifically bind to the RNA transcript and transiently modify its processing mainly through RNAse-mediated degradation, transcript stabilization, altered splicing or blocked translation. Possible therapeutic approaches in haplo-insufficiency-related diseases include upregulation of the expression from the wild-type allele. Molecular mechanism of β-catenin reveals that when Wnt signalling pathway is off, the majority of β-catenin is destined for degradation through the action of the destruction complex that binds, phosphorylates and ubiquitinates β-catenin which in turn undergoes proteasomal degradation. We sought to upregulate WT β-catenin by rescuing it from the pool destined for degradation through skipping of exon 3 that contains motifs, important for β-catenin degradation. In order to check if such deletion indeed results in β-catenin stabilization while retaining all of its functions we first prepared and analysed the target exon deletion construct (β-catenin$^{\Delta ex3}$). Upon its transfection to HEK293T cells in combination with TopFlash reporter we confirmed that it was expressed and possessed same Wnt transcription activity as WT β-catenin, while split YFP interaction assay revealed that it also comparably interacted with cadherin. We proceeded to

design several ASOs to induce skipping of target exon and screened them in HEK293T. Transfection of the best ASO candidate induced robust exon 3 skipping which was not observed when testing its scrambled version. It also led to the expression of truncated β-catenin, upregulated Wnt activity as measured by TopFlash reporter assay as well as significantly upregulated expression of Wnt targeted genes as measured by qPCR. We were able to reproduce these findings in human neuroblastoma cell line. We also detected ASO-mediated exon skipping in CTNNB1 syndrome patient-derived iPSC cells and cortical organoids following gymnotic uptake of the ASO which uniformly distributed throughout the organoids. Finally, stereotactical injection of the ASO in combination with TopFlash reporter into WT mouse brain lead to the increase in Wnt transcription activity. In conclusion, we successfully exploited CTNNB1 haplo-insufficiency and β-catenin degradation mechanism in order to design ASO-based approach for β-catenin supplementation which has the potential to be universally applicable to all CTNNB1 syndrome patients, regardless of the mutation type and location.

P0336

Ectopic expression of two vertebrate cone opsins in retinal ganglion cells results in opposite responses to light stimulation, which is likely determined by selective G protein activation

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Retinitis pigmentosa is a genetically heterogeneous group of diseases causing retinal degeneration and blindness. In the past twenty years, microbial opsins (light-sensitive ion channels and pumps from bacteria or algae) have been shown to successfully restore partial vision when expressed in retinal ganglion cells (RGCs) of retinitis pigmentosa patients. However, microbial opsins are inherently limited by their low light sensitivity and their potential immunogenicity. As a next generation therapy, vertebrate opsins (such as melanopsin, rhodopsin and cone opsins) have been proposed and tested in rodent models to improve upon these two weaknesses. One study used mid-wavelength sensitive cone opsin (MW-opsin) to activate mouse RGCs through an unknown endogenous G-protein. To better understand coupling between opsins and the G protein-coupled signalling in mouse RGCs, we expressed the short-wavelength sensitive mouse cone opsin (Opn1sw) in the same cells. RGC responses were measured *ex-vivo* using multielectrode array and patch clamp. Surprisingly, Opn1sw created an opposite, hyperpolarizing effect on membrane potential of RGCs suggesting a different signalling pathway. Single cell RNA sequencing showed significant differences in the G protein profile of RGCs and cone photoreceptors. This consolidates our hypothesis that different cone opsins might activate different G protein pathways when ectopically expressed in the same cell type, triggering different types of responses through different final effector channels. Overall, our results indicate a need to better understand the intricate relationship between cone opsins and their signalling cascades within different cells in the retina. Such understanding will lead to the creation of better and more efficient opsins, leading to second-generation therapies.

P0337

Characterization of retinal degeneration in patients with rod-cone dystrophy to inform on potential efficacy endpoint of gene therapy

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Rod-cone dystrophy (RCD) is a rare inherited retinal disorder leading to significant vision loss, and for which no treatment is currently available to most patients. Photoreceptor degeneration progresses unevenly and slowly over decades, making it hard to assess investigational drugs aiming at delaying or stopping photoreceptor degeneration. PHENOROD2 (NCT04285398) is an ongoing prospective natural history study assessing RCD progression, to help select relevant clinical efficacy endpoints for the evaluation of novel therapies like SPVN06, a gene-independent investigational gene therapy that aims to slow down the progression of central vision loss in patients with RCD, regardless of the underlying pathogenic variant(s).

Adult patients with RCD caused by a variant in the *RHO*, *PDE6A*, or *PDE6B* gene were enrolled in Paris, France and Pittsburgh, PA. At inclusion, visual acuity had to be ≥ 20/200 in at least one eye, and binocular visual field ≥ 5°. Retinal anatomy was imaged by spectral-domain optical coherence tomography (Spectralis, Heidelberg) and short-wavelength fundus autofluorescence (Heidelberg Retina Angiograph HRA2), at inclusion and Year 1. Disease progression was defined as a loss at Year 1 greater than -110 µm or -9% in either the horizontal width of the ellipsoid zone (hEZ), or the horizontal or vertical diameters of the inner ring of autofluorescence (hFAF or vFAF).

A total of 80 patients were assessed at Year 1. At inclusion, mean (SD) age was 47.4 (12.8) years and patient-reported disease duration ranged from 5 to 61 years. On average, hEZ was 1939 (1267) µm, hFAF was 1886 (808) µm, and vFAF was 1530 (680) µm. At Year 1, mean change from baseline was -105.6 (121.6) µm for hEZ, -108.5 (147.6) µm for hFAF, and -85.2 (125.7) µm for vFAF, representing a mean loss of -7.4 (9.5) %, -6.7 (9.1) %, and -6.9 (10.1) %, respectively.

A ring of autofluorescence was distinguishable in only 80 (50%) eyes, making it a parameter of lesser relevance to monitor disease progression. Consequently, this analysis focused on the hEZ parameter, a structural marker more commonly used as an endpoint in clinical trials of retinal degeneration, and recently accepted by the FDA as an approvable endpoint in dry AMD. Among the 130 eyes for which a change from baseline of hEZ was available, 66 (51%) had progressed at Year 1, representing 52/70 (74%) patients; and 23 (18%) eyes showed a loss of more than twice the threshold of progression, representing 21 (30%) patients.

After one year of prospective follow-up, markers of retinal degeneration showed a significant change in a majority of patients with RCD, although with high heterogeneity. This analysis allowed the identification of subjects whose disease is progressing more rapidly, and who can be of interest for their participation in the ongoing first-in-human trial of SPVN06, PRODYGY (NCT05748873).

P0338

AAV capsid discovery in the cochlea of non-human primates for precision gene medicine of congenital auditory indications

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Hearing loss is the most common sensory impairment in the population, with more than 400 million people affected worldwide. More than half the cases of non-syndromic profound congenital hearing loss have a genetic cause, from which approximately 80% are autosomal recessive, with a prevalence of about 1-2 every 1000 births. Currently, cochlear implants are the standard of care treatment, and gene therapy emerges as an alternative to cure congenital hearing loss disorders.

Adeno-associated viral vectors (AAVs) have become the flagship delivery vehicle for *in vivo* gene therapy and some AAVs, especially novel bioengineered capsid variants, have shown remarkable efficiency transducing cochlea cells in preclinical models. Nevertheless, the cochlea is a highly specialized multicellular organ that requires fine-tuning of both targeting and specific expression from the gene therapy vehicle to achieve long-term therapeutic effect and avoid undesired vector-related toxicity. In order to identify AAV capsids with narrow tropism for cochlea cells, we have screened a library of 68 AAV naturally occurring and synthetic variants in the cochlea of non-human primates. Each variant of the library encodes a CMV-eGFP cassette with a unique barcode upstream the polyA, allowing NGS-barcode analysis at both the DNA and RNA levels. The AAV kit was dosed into the cochlea through the round window membrane that was accessed by a transmastoid tympanotomy. The procedure and the dosing were well tolerated. Fourteen days upon administration, fresh samples from the injected cochlea as well as additional tissues were taken for NGS and histological analyses. From the 68 AAV variants administered locally, forty transduced cochlea cells efficiently, but only 22 targeted the cochlea exclusively from which we selected 6 candidates based on the barcode RNA expression. Fifteen variants were found in different brain regions, auditory nerve and dorsal root ganglia (DRGs), showing eGFP expression in DRG neurons and the choroid plexus. Interestingly, eGFP expression was also found in the spleen, where more than 20 AAV variants were detected by NGS. The selected novel variants were used to generate a new library that also included five control variants. The sub-selection of variants was dosed in another NHP cochlea following the same approach. NGS and IHC confirmed that our selection highly restricted EGFP expression of the variants to cochlea cells compared to other tissues, and we are currently cross-validating the cell tropism of the selected AAV variants in the adult mouse cochlea.

In summary, the AAV kit screening in the NHP cochlea allowed us to identify AAV capsids that exclusively targeted and expressed the reporter transgene in the organ of interest. Current analyses will confirm cell specificity within the cochlea in order to design precision gene therapies for the different types of congenital hearing loss disorders.

P0339

Nonclinical evaluation of CTx001, a gene therapy for the treatment of geographic atrophy in aged-related macular degeneration

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Age-related macular degeneration (AMD) is strongly associated with genes that regulate the alternative complement pathway. Complement Therapeutics is developing CTx001, an AAV gene therapy expressing a soluble, truncated form of the complement regulatory protein complement receptor 1 (mini-CR1) for the treatment of geographic atrophy (GA).

CTx001 was produced at 50L scale via triple transfection methodology in HEK293 cells. In vivo activity of CTx001 was verified in the rat laser CNV model before progressing to NHP studies. Tolerability of CTx001 in NHP was assessed following a single, bilateral administration of drug into the subretinal space of cynomolgus macaques at low, medium and high dose, followed by an eight week observation period. Volume matched vehicle control delivered via the same route of administration was used as comparator. Study endpoints included clinical observations, clinical pathology, biomicroscopic and funduscopic ophthalmic exams, tonometry, SD-OCT and fluorescein angiography.

CTx001 displayed bioactivity in vivo, attenuating activation of the terminal complement pathway in the rat laser CNV model. CTx001 was well tolerated in NHP at all dose levels. All animals progressed to scheduled euthanasia, and there were no gross lesions apparent upon necropsy. There were no test item related clinical observations reported throughout the study, and animals gained and maintained weight consistent with vehicle controls. No remarkable findings linked to test item were seen in clinical chemistry, hematology or coagulation parameters. Mild inflammation secondary to the dosing procedure and test item was correlated with increased severity in the high-dose group. Ophthalmic findings such as pigment variation, anterior flare, vitreous and retinal hemorrhage were all reported as very slight to slight. There were no signs of retinitis in any dose groups. Finally, CTx001 protein transgene accumulated in the NHP vitreous, was bioactive and correlated with biomarkers of target engagement

In this eight week, non-GLP dose range finding study, CTx001 produced at clinical scale maintained bioactivity in vivo and was well tolerated following subretinal administration in NHP up to maximal feasible dose. These results support initiation of IND enabling GLP toxicology studies for CTx001.

P0340

Exploring miR-181a/b inactivation as a novel therapeutic strategy for Glaucoma: enhancing Retinal Ganglion Cells survival through mitochondrial protection

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Glaucoma, a leading cause of global blindness, is a multifactorial disorder characterized by loss of Retinal Ganglion Cells (RGCs) and optic nerve damage. Although this disorder's pathological mechanisms are still largely unknown, age, genetics, and elevated intraocular pressure (IOP) represent prominent risk factors. However, one-third of cases have optic nerve degeneration despite IOP. To date, pharmacological strategies are focused on reducing IOP, which is unfortunately ineffective in most cases.

The similarities between glaucoma and Mitochondrial Optic Neuropathies (MON), have driven a growing interest in exploring mitochondrial function in glaucoma. In particular, mtDNA mutations, decreased mitochondrial respiration, and oxidative stress have been described in patients and in animal models and have been linked to glaucoma pathogenesis, thus suggesting that mitochondria may represent a possible therapeutic target.

We recently demonstrated that the microRNAs 181a and b (miR-181a/b) regulate mitochondrial biogenesis, function, and ROS detoxification in the retina. Moreover, we found that miR-181a/b downregulation protects RGCs and ameliorates visual function in different mouse models of MON, indicating that these microRNAs could represent attractive therapeutic targets for glaucoma.

We thus tested the therapeutic effects of miR-181a/b downregulation in either the optic nerve crush (ONC) injury mouse model and in DBA/2J mice representing normal-tension and high-tension glaucoma, respectively. ONC was performed in 3-month-old *miR-181a/b*$^{+/+}$ and *miR-181a/b*$^{-/-}$ mice. Six-month-old DBA/2J mice were intravitreally injected with Adeno Associated Viruses (AAV) 2/2 encoding a miR-181a/b "sponge" in one eye and AAV2/2-GFP in the contralateral eye as a control. The analysis of the phenotype was performed at different time points (post-ONC or AAV injection) by histological analysis to assess the number of RGCs and by the analysis of the Photopic Negative Response (PhNR), a component of Electroretinogram linked to RGC activity, to test the visual function.

Interestingly, our preliminary findings showed a significant amelioration of the disease phenotype in both ONC and DBA/2J models following miR-181a/b downregulation, demonstrating a powerful neuroprotective effect. Moreover, enhanced RGC survival, improved visual response, and structural amelioration of the optic nerves were evident in DBA/2J mice injected with AAV2/2-miR181a/b sponge with respect to controls. Ongoing analysis of the axonal density will better clarify the effect of miR-181a/b modulation on optic nerve integrity.

Overall, our data suggest miR-181a/b as a new therapeutic target for both normal- and high-tension glaucoma. Targeting mitochondrial dysfunction represents a novel strategy to mitigate RGC loss and alleviate vision impairments. Moreover, our findings may, in the long run, provide a

novel RNA-based therapeutic product that could eventually be translated into the clinic, offering a potential solution applicable to different forms of glaucoma.

P0341

Rescuing *SCN1A* haploinsufficiency by translation enhancement: a novel base editing approach for Dravet syndrome

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Dravet syndrome (DS) is a severe developmental and epileptic encephalopathy arising in the first year of age with drug-resistant febrile seizures, later accompanied by developmental, intellectual and behavioral abnormalities. Up to 20% affected individuals die before reaching adulthood, frequently due to sudden unexpected death in epilepsy. In more than 80% of patients, DS is caused by haploinsufficiency of *SCN1A* gene, encoding for the alpha subunit of the voltage-gated sodium channel $Na_V1.1$. $Na_V1.1$ is mainly localized at the axonal initial segment of GABAergic inhibitory interneurons where, its reduced expression in DS, causes a reduced excitability, ultimately leading to neuronal circuitry imbalance. Up to now, there is no definitive cure for DS. Ongoing strategies enhancing *SCN1A* gene expression at a transcriptional level have been developed; nevertheless, they require the permanent overexpression of a transcriptional activator that represents the major hurdle towards clinical translation. Here we describe a novel gene therapy strategy exploiting permanent CRISPR-Cas9 base editing of 5' untranslated (UTR) regulatory sequences of mRNA to boost $Na_V1.1$ protein translation.

First, to identify candidate elements in the 5' UTR of *Scn1a* mRNA potentially impacting its translation, we generated a reporter system screening platform. Starting from the wild-type sequence, we cloned 22 different variants of *Scn1a* 5'UTR harboring distinct combinations of nucleotide transitions in a GFP/mCherry bicistronic reporter vector. In such system, GFP translation is directly dependent on putative 5'UTR variants, while mCherry works as internal normalizer. FACS analysis was exploited to measure GFP intensities across variants. Two out of 22 variants, sharing the same mutation combinations, were able to significantly increase GFP fluorescence intensity of 2-fold over wild-type sequence. This evidence confirms the presence of putative elements in the 5'UTR of *Scn1a* mRNA attenuating its translation that, when mutagenized, lead to a significant boost in protein levels. To reproduce best variants mutations in the endogenous *Scn1a* locus, we set SpRY and/or SpCas9 base editing strategies in HEK293T cells. Among the sgRNAs tested, two displayed 30% on-target editing efficiency, resulting in the effective reproduction of the 5'UTR mutations in the endogenous locus. To test whether the editing of translation regulatory sequences increased the endogenous $Na_V1.1$ protein levels, we next delivered base editors and respective sgRNAs in cortical and hippocampal mouse primary neurons cultivated *in vitro* via AAV and Lentiviral vectors. Interestingly, we were able to install on-target edits with similar efficiency as observed in cell lines, that however demonstrated to be sufficient to significantly increase $Na_V1.1$ protein, with no effect on mRNA levels.

Overall, our preliminary data support the feasibility of augmenting $Na_V1.1$ protein levels by installing single-base conversion within endogenous *Scn1a* 5'UTR translation-regulative elements. Such strategy represents a new way of rescuing haploinsufficiency in DS patients with three major advantages: a) avoiding life-long administrations; b) being universal for all patients regardless of causing pathogenic mutations; c) possibility to be combined with *SCN1A* transcriptional or splicing enhancing therapies.

P0342

Retinal microglia culture as a screening platform of novel AAV variants for efficient gene delivery

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Microglia are the resident immune cells in the central nervous system (CNS) and retina. They are involved in tissue homeostasis, particularly immunosurveillance and neurogenesis, but also disease. Indeed, microglia dysfunction and mutations of microglia-specific genes have been reported to play a key role in the pathobiology of neurodegenerative disorders. Consequently, these cells have recently become the focus of new treatment approaches. Although most studies on microglia refer to the CNS, it is known that the brain and the retina microenvironments are unique and determine differences in microglia function and profile between organs. The characterization of retinal microglia is limited due to the restricted availability of cells and the lack of efficient primary culture methods that allow long-term and adequate cell numbers. Moreover, its manipulation has been hindered by the difficulty of targeting these cells using viral and non-viral modalities. To overcome these major challenges, here we introduce a novel microglia culture protocol from mouse retina, which is reproducible, and is characterized by high cell numbers and long *in vitro* viability. This new culture system comprises highly pure retinal microglia cells that remain viable for at least one month and retain the ability to perform phagocytosis. We used this model as a screening platform to evaluate CMV-mediated reporter transgene expression of engineered adeno-associated virus (AAV) vectors AAV6- or AAV1-derived capsids, the most used serotypes on glial cells. The variants carried single-point mutations of surface-exposed tyrosine, lysine, threonine, serine residues, and/or arginine residues, or the peptide insertions of AAV2.GL and AAV2.NN, two previously described 12-mer peptide insertions that enhance retinal cell transduction when introduced into the AAV2 capsid. The variants with the GL and GL.R modifications showed an increased transduction efficiency of primary mouse retinal microglia cells. In particular, the variants based on the AAV1 capsid showed transduction efficiencies that were more than 10-fold higher than the AAV1 wild-type and around 2.5-fold higher than the triple mutant AAV6 TM6 at 5 dpi. This improved activity has been confirmed in both resting and chemically activated microglia and allowed us to achieve dCas9-VPR (epi)genome editing to transactivate endogenous genes in the targeted cells. Finally, we started to validate our variants in mouse models of retinal disorders. In the Rd1 mouse model, which shows a strong induction and activation of microglia, we were able to reach 20% of Cd11b+Cd45+ cells after intravitreal injection of AAV1.GL or AAV1.GL.R. Overall, our newly defined conditions facilitate functional and molecular studies in poorly investigated retinal microglia. Recently, we were able to obtain a comparable microglia population from human retinal donor tissue samples displaying a characteristic immune-related marker and proteomic profile. We propose this retinal microglia culture model as an innovative *in vitro* platform for assessing novel AAV capsids, gene therapies and genome editing approaches for efficacy/potency.

P0343

Metabolomic profiling approach to identify peripheral blood changes after AAV-CYP46A1 intrastriatal injection in HD mice

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In Huntington's disease (HD), impaired brain cholesterol homeostasis is a critical driver of HD progression. The key neuronal enzyme, cholesterol 24-hydroxylase (CYP46A1), is declined in HD patients' brains. CYP46A1 reduces excess cholesterol in neurons, by regulating cholesterol biosynthesis and producing 24-OH Cholesterol that can cross the blood-brain-barrier and is eliminated in the periphery. In HD, reduced CYP46A1 is associated with cholesterol accumulation in neuronal membranes impairing the clearance of aggregated mutant Huntingtin, vesicular transport and synaptic transmission, leading to death of neurons (Kacher 2022) in HD mice. Restoring CYP46A1 using an adenovirus associated virus (AB-1001) (AAV)(Samulski et al 1982) , reinstates cholesterol metabolism in striatal neurons and mitigates HD neuropathological hallmarks (Boussicault 2016). By restoring the cholesterol metabolism, CYP46A1 overexpression may have general consequences on cellular metabolism that could translate into changes of key metabolic compounds in the injected tissue and plasma of AAV-CYP46A1-treated HD mice. Using a metabolomic approach, this study aims to identify specific modification of the metabolic profile in the brain of AAV-CYP46A1-treated HD mice.

We first aimed to evaluate the performance of different LC/MS columns in which both hydrophilic interaction liquid chromatography (HILIC) and perfluorophenyl (pFPP) columns were compared to determine which one provides better coverage and detection of (modified) metabolites after AB-1001 delivery. Wild-type and R6/2 HD male mice (n=5-10/group) received bilateral striatal injections and were sacrificed 6 weeks post-dosing: R6/2 treated with AAV-CYP46A1, vehicle or non-injected and wild-type treated with vehicle or non-injected mice were used. Biological samples (striatal and plasma samples) were collected 6 weeks post-dosing. Subsequent data processing involved peak detection and compound annotation using libraries. Multivariate statistical analyses were conducted to compare the performance of the two columns in terms of metabolites coverage and detection.

The comparative analysis revealed distinct metabolites profiles for each column. The HILIC column excelled in detecting highly polar metabolites while the low number and the range of differential metabolites obtained in pFPP columns could not be interpreted alone without the patterns observed in HILIC conditions.

Further experiments focusing on the HILIC column were carried out on a second cohort of male and female HD wild-type and R6/2 mice (n=5-10/group) who received bilateral striatal injections. The refined HILIC data revealed a metabolic profile modified by treatment, facilitating future comparison with metabolomic data from HD patients.

Altogether, we believe this study paves the way for the establishment of candidate metabolite biomarkers using LC-HRMS after AAV-CYP46A1 gene therapy.

P0344

Improving gene editing for inherited retinal dystrophies: HDR correction by CRISPR and TALEN in patient-derived iPSCs rescues disease phenotype

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Inherited retinal dystrophies (IRD) are a group of rare disorders triggering blindness and other ocular complications that currently have no effective treatment. They are caused by pathogenic variants in near 300 genes—in the majority of cases point mutations— that result in retinal cells degeneration.

Gene editing postulates as a promising and powerful strategy for the development of IRD therapies allowing the permanent correction of pathogenic variants. By using CRISPR and TALEN technologies we aimed to precisely correct IRD-related mutations in seven iPS cell lines derived from patients with Retinitis Pigmentosa, Stargardt disease, Best disease or Achromatopsia.

We achieved correction of all the hiPSCs by either CRISPR/Cas9 or TALEN approximations without detected off-target effects. In most assays, at least 50% of the clones exhibit pathogenic variant repair without on-target aberrations, reaching a maximum gene editing efficiency of the 80%. The best results were obtained in the correction of the small insertion, deletion and homozygous mutations. Surprisingly, the repair of heterozygous carriers rendered, in few cases, edited clones harbouring the pathogenic variant in homozygosis, which suggested DNA double-strand break (DSB) of the wild-type allele and repair by using the mutant allele as template for correction. Thus, sgRNA specificity for the mutant allele decisively influences gene editing outcomes, and prevent undesired DSB of the wild-type DNA. Taking this into account, we were able to precisely correct pathogenic variants without transfecting the exogenous template, showing DSB repair by using the endogenous homologous DNA of the sister chromatid.

Edited cells were then differentiated *in vitro* into several cellular models of the retina, such as photoreceptors, retinal pigment epithelial cells (RPE) and retinal 3D models (organoids), to analyse the potential reversion of disease-associated phenotypes. Several analyses in corrected clones of different hiPS cell lines exhibit improved retinal cells differentiation and function compared to isogenic mutant clones, and similar to that observed in wild-type hiPSCs indicating the reversion of the disease condition.

Gene editing represents a promising approach for IRD treatment with CRISPR/Cas9 postulating as a more robust technology but TALENs being appropriate in cases where CRISPR not due to genomic concerns. These results show efficient and precise correction of pathogenic variants without undesired genomic aberrations, and strengthen the study and application of gene editing as a therapeutic approximation for IRD. Moreover, these data suggest an unexpected potential application of CRISPR as a donor-template-free strategy in the correction of heterozygous pathogenic variants by using patient's own wild-type allele. Last, isogenic clones obtained through gene editing allow disease modelling contributing notably to unravelling the role and function of IRD-related genes.

P0345

Efficient retrograde transport following stereotaxic inoculation in the striatum of a non-replicative HSV-1 vector

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Projection neurons send axons to distant targets. Efficient retrograde access to projection neurons for the delivery of a specific transgene represents a major advantage for gene therapies targeting neurodegenerative disorders. Recombinant adeno-associated viruses (AAVs) have become the reference for in vivo gene delivery in the central nervous system (CNS). However, their natural retrograde transport ability is low, limiting efficient targeting of projecting neurons. Moreover, AAVs have limited packaging capacity and typically require high intraparenchymal doses (10^9 to 10^{12} particles).

Herpes simplex virus type 1 (HSV-1) is a human neurotropic virus that establishes productive infections in epithelial cells and latent infections in neurons. Non replicative (nr) HSV-1 based vectors have been developed to exploit their therapeutic potential by leveraging their biodistribution, safety, and long-term transgene expression abilities. In contrast to AAVs, nrHSV-1 vectors have large packaging capacity, can efficiently transduce neurons of the central nervous system at low doses, and can be transported retrogradely or anterogradely to connected brain regions. However, results obtained by different teams with nrHSV-1 are not always consistent, even when inoculated in the same region, probably reflecting that subtle vector differences, such as the strain of the virus used or its precise genetic composition can significantly affect biodistribution or expression within the brain.

We constructed a nrHSV-1 vector (EG133A) with deletions in the essential ICP4 and ICP27 genes. This vector conserves a single copy of ICP0, an HSV-1 regulatory gene, and expresses the mGreenLantern reporter gene under the control of EF1a promoter. When stereotaxically injected into the striatum of mice with only 4E+06 PFU (Plaque Forming Unit), EG133A demonstrated high-level transgene expression in neurons at the injection site. It was also efficiently retrogradely transported to dopaminergic neurons in the substantia nigra pars compacta, thalamus, basal ganglia, and prefrontal cortex, where similar transgene expression was observed. Co-staining with Tyrosine Hydroxylase showed that up to 40% of dopaminergic neurons in the substantia nigra expressed the transgene after a single injection. Only few astrocytes and microglial cells were transduced at the injection site, demonstrating high neuronal specificity of the vector. This distribution pattern was observed at both 3- and 10-days post-injection. Histopathology at 3 days in animals administered with EG133A showed only mild inflammatory findings at the administration site, consisting of focal presence of inflammatory cells and mild gliosis, compared to vehicle-injected animals.

Our results suggest that intra-striatal administration of low amount of EG133A efficiently targets neurons at the administration site and projecting neurons by retrograde transport, with a favorable safety profile. This vector is a promising tool for gene therapies targeting both dopaminergic and cortical neurons.

P0346

Differential hearing restoration in the DFNB9 mouse model through AAV gene therapy with human and mouse cDNA

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Hearing loss is the most common sensory deficit in humans and a major concern for Public Health issue as it impacts everyday life, causing loneliness, isolation, dependence, as well as communication disorders. It is currently affecting approximately 430 million people worldwide. The clinical prevalence of congenital deafness is approximately 1 in 700 newborns, 80% of these cases are attributed to a genetic cause. Deafness caused by mutations in the otoferlin gene, known as DFNB9, accounts for 2 to 8% of genetic deafness cases.

Our team developed and characterized a DFNB9 murine model, demonstrating that otoferlin is crucial for the final steps of synaptic exocytosis, ensuring ultrafast vesicular neurotransmitter release at inner hair cell (IHC) ribbon synapses. This finding led to the conclusion that DFNB9 deafness is a genetically linked auditory synaptopathy. Notably, the team developed a therapeutic vector that provides the first proof of concept that viral gene therapy can effectively treat hearing impairment in a preclinical murine model of human DFNB9 deafness. The human version of this therapeutic vector is currently in successful phase II clinical trials for DFNB9 patients. One of the main unresolved questions in both fundamental and clinical research is whether the preclinical mouse model treated with DFNB9 gene therapy achieves similar hearing recovery and sound signal processing regardless of whether the administered vector delivers human or mouse cDNA.

To address this issue, we administered either sequence at two different time points, before (P2) and after hearing onset (P15) using a dual-AAV approach. Thereafter, hearing function was evaluated at different time points, using auditory brainstem responses (ABR), startle reflex, and pre-pulse inhibition (PPI). Following auditory screening, the correct expression and localization of both proteins in the cochlea were confirmed with immunohistochemistry. Our results using murine cDNA validated previous results obtained with gene replacement therapy for DFNB9: ABR thresholds were restored to wildtype levels and the startle reflex and PPI were fairly rescued. Conversely, we found that treatment with human cDNA was not sufficient to elicit the same degree of auditory recovery, ABR thresholds were higher, wave I amplitudes were lower, and latencies were increased. Similarly, we observed an impaired startle reflex and absence of PPI when using the human cDNA. These results suggest that there is an intrinsic functional deficit of human Otoferlin protein when expressed in the murine cellular environment. This hypothesis was supported by IHC membrane capacitance measurements, demonstrating that Ca^{2+}-dependent synaptic exocytosis was only partially recovered after treatment with human OTOF. In contrast, as expected, Ca^{2+}-dependent synaptic exocytosis was almost fully restored after treatment with murine Otof. To understand how the observed differences at the first sensory synapse influence central sound processing, we are currently performing in-vivo high-density electrophysiological recordings in two major relay stations of the auditory pathway (the cochlear nucleus and the inferior colliculus). This study would elucidate how gene therapy at the peripheral level can restore essential features of sound processing at the central level.

P0347

A Multimodal Preclinical Platform for Personalized Testing of Antisense Oligonucleotide Therapeutics in Neurodegenerative Diseases

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Antisense oligonucleotides (ASOs) are short RNA sequences that bind to complementary (pre-) mRNA in the cytosol or nucleus and alter the processing of the target gene by regulating splicing or initiating degradation through RNase H recruitment. Current methods for developing ASO therapies for neurodegenerative diseases lack a human-relevant and scalable testing platform and rely heavily on animal models.

We aim to establish a multimodal platform using iPSC-derived neurons and brain organoids to evaluate the efficacy, longevity, specificity, and toxicity of ASOs in the genetic background of patients in a disease-specific context, focusing on spinocerebellar ataxia type 3 (SCA3). SCA3 is an inherited neurodegenerative disease caused by a CAG repeat expansion within the coding region of *ATXN3*. While the pathogen-associated protein has an increased tendency to aggregate, the wild-type protein performs essential functions as a deubiquitinase. ASOs could specifically target disease-associated variants of the mutant (pre-)mRNA and thus reduce the toxic protein concentration while preserving the wild-type protein.

We optimized a workflow to identify disease-associated SNPs using a multiplexed, targeted allele-specific sequencing approach. Gapmer ASOs targeting different disease-associated SNPs were screened in iPSC-derived neurons and further validated in cerebellar organoids from SCA3 patients. Several gapmer ASOs were identified that are able to specifically reduce mutant ataxin-3 protein levels in both iPSC-derived neurons and cerebellar organoids, while maintaining the expression of the wild-type form. Cerebellar organoids will be used for in-depth characterization of promising ASOs, including acute and long-term toxicity assays and analysis of on- and off-target effects, to enable improved, personalized and patient-centric analysis of ASOs as a treatment for SCA3.

iPSC-derived neural models provide a human-relevant platform to evaluate ASOs for neurodegenerative diseases. This platform will allow the assessment of ASO efficacy, off-target effects, acute and long-term toxicity using protein quantification, calcium imaging, NfL levels, and omics technologies. Once established, this platform is adaptable to many diseases affecting different parts of the brain and for the evaluation of other RNA-based therapeutics.

P0348

Dynorphin-based gene therapy for treatment of temporal lobe epilepsy

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With a worldwide prevalence of around 1% epilepsy represents the most common chronic CNS disease and temporal lobe epilepsy (TLE) is the most frequent clinical presentation. In TLE the epileptogenic focus mostly lies in the hippocampus, involved in learning memory and emotional control. The high incidence of drug resistance, devastating comorbidities, and unpredictable responsiveness to invasive epilepsy surgery pose unmet medical challenges.

In the quest of novel, disease-modifying treatment strategies neuropeptides represent promising candidates. We have provided proof of concept that AAV-mediated delivery of dynorphins into the epileptogenic focus leads to nearly complete and lasting suppression of seizures in the clinically accepted intrahippocampal kainic acid mouse model of TLE. Importantly, release of dynorphins is excitation-triggered, dependent on high-frequency stimulation, as at the onset of seizures (Agostinho et al, EMBO Mol Med 2019, e9963). Furthermore, the debilitating decline of learning and memory is prevented. In human hippocampal slice culture obtained after epilepsy surgery, AAV-delivered dynorphins are expressed and released, and dynorphin suppresses seizure-like activity.

To prepare for a first in man study, AAV vector backbone and transgene delivery were optimized, and scale-up vector manufacturability confirmed. This leads to an enhanced lead vector with potent anti-seizure activity in animal models of chronic TLE. Furthermore, we have set-up *in vitro* potency assays to quantify AAV-transduced dynorphin. Dose finding studies and an extended panel of QC-assays are currently being performed as part of ongoing IND-enabling studies.

The novel format of “release on demand” delivery of dynorphin peptides for treatment of TLE is viewed as a key to prevent habituation and to minimize the risk of adverse effects, together allowing long-term suppression of focal seizures and of their devastating sequels.

P0349

iPSC derived cardiomyocyte disease modeling of Friedreich’s Ataxia and Duchenne Muscular dystrophy for therapeutic assessment

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The reprogramming of patient-derived fibroblasts into induced pluripotent stem cells (hiPSCs) and the successful differentiation of these cells into cardiomyocytes (hiPSC-CMs) has opened the

possibility to model human disease in a dish. Here we present data for models of Friedreich's ataxia (FRDA) and Duchenne Muscular Dystrophy (DMD) in a platform with closer fidelity to human pathophysiology, recapitulating, at the cellular level, more aspects of disease phenotype than murine or other animal models.

Control and diseased (FRDA and DMD) hiPSC-CMs were differentiated using controlled stirred-tank bioreactors which enables large-scale production with regulated culture conditions (e.g., pH and gas exchange). This minimizes batch to batch variation and results in high yield production. Sufficient quantities of consistently pure hiPSC-CMs were produced in one run, allowing use in multiple drug screens.

The underlying cause in FRDA is a chronic reduction in frataxin levels, while in DMD it is the lack of dystrophin. Therefore, we first set out to evaluate levels of frataxin and dystrophin by utilizing an ELISA assay and immunocytochemistry (ICC), respectively. As expected, the cells from the FRDA patient line showed significantly lower amounts of frataxin protein compared to cells from the healthy control line, while the DMD iPSC derived cardiomyocytes showed no expression of endogenous dystrophin.

A hallmark of the FRDA disease phenotype is elevated levels of ROS. Using FACS with a CellRox probe, we observed a significant increase in ROS levels in FRDA hiPSC-CMs compared to control.

For both FRDA and DMD diseases, abnormalities in the calcium handling of cardiomyocyte cells is observed. After recording the calcium transients of FRDA and control hiPSC-CMs using the FDSS/µCell kinetic reader (Hamamatsu), we found that the calcium transient duration and decay were significantly prolonged in FRDA hiPSC-CMs.

For DMD hiPSC-CMs the calcium transients and contractility (via impedance measurements) were recorded and analyzed. We observed a significant decrease in calcium transient amplitude and calcium transient duration of DMD hiPSCs-CMs compared to healthy controls.

To ameliorate the adverse effects seen in the functional phenotype of DMD and FRDA hiPSC-CMs, the cells were transduced with an AAV carrying Frataxin or Dystrophin. The ELISA assay confirmed the successful overexpression of frataxin and ICC confirmed the overexpression of dystrophin. We observed a significant decrease both in the calcium transient duration and in the ROS levels of FRDA hiPSC-CMs while in the DMD lines, we observed significant recovery of the calcium amplitude.

In conclusion, the reprogramming of patient-derived fibroblasts into hiPSCs and their differentiation into cardiomyocytes provides a powerful platform for modeling human diseases, such as FRDA and DMD. Using controlled stirred-tank bioreactors for large-scale production, we achieved high yield and consistent purity of hiPSC-CMs, enabling extensive screening. Ncardia's evaluations confirmed the expected deficiencies in frataxin and dystrophin in FRDA and DMD models, respectively, and observed specific disease-relevant phenotypes. Importantly, we demonstrated the potential of this platform for therapeutic assessment and development by evaluating phenotype rescue after introducing AAV vectors with Frataxin or Dystrophin.

P0350

Introducing the National Facility for Genome Engineering and Disease Modelling (NF GEDI)

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The introduction of methods for generating induced Pluripotent Stem Cells (iPSCs) from somatic cells has unlocked new opportunities in human disease modelling, drug discovery, and regenerative medicine. The capacity to differentiate iPSCs into various tissue types preserving 3-dimensional organization and cell diversity as well as the introduction of gene editing, marked the onset of a new era for human preclinical research. Nevertheless, these methodologies require time, state-of-the-art infrastructures, and training of specialised personnel. The **National Facility for Genome Engineering and Disease Modelling (NF GEDI)** aims to support the scientific community by offering specialized resources and scientific support in stem cell biology, disease modelling, and genome engineering. GEDI offers off-the-shelf services such as iPSC **reprogramming,** 2-D/3-D cell **differentiation,** and **genome engineering**. Moreover, the Facility supports the development and optimization of innovative methods and provides training and educational programs. From 2024, the NF GEDI will open its services to the national scientific community, with the long-term goal of becoming a national hub for stem cell biology and disease modelling. By combining sophisticated methodologies with the implementation of robotic platforms, the Facility will bridge the gap between small- and large-scale experiments, which today hinders long-term planning and realisation of ambitious projects. Our ongoing commitment is to implement state-of-the-art infrastructure and cutting-edge technologies, ensuring a cost-effective advancement of the national research in the field and bringing Italian researchers to compete worldwide.

P0351

AAV-mediated gene therapy attenuates loss of vision in a mouse model of Bardet-Biedl-Syndrome 10

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Bardet-Biedl syndrome (BBS) is a group of inherited, autosomal recessive ciliopathies characterised by disturbances of cilia function in multiple cell types, leading to obesity, renal failure, and blindness. More than 20 causative genes are known with many mutations disabling the function of the BBSome, a protein complex regulating the movement of cargo proteins in and out of cilia. Mutations in the *BBS10* gene are the second most common cause of BBS and account for more than 20% of all cases. Recently, a proof-of-concept study by Hsu et al. 2022, demonstrated the potential of subretinally delivered AAV gene therapy using mouse *Bbs10* in a *Bbs10*-deficient mouse model. In this study we set out to optimise and identify an AAV vector carrying the human *BBS10* gene providing sustained efficacy and a good safety profile for clinical translation.

Human *BBS10* either under the control of the ubiquitous CAG promoter or the photoreceptor-specific rhodopsin kinase (RK) promoter was packaged into AAV8 and tested in *Bbs10* KO mice at different doses. Whilst the CAG construct did not show efficacy, treatment with the RK construct rescued retinal function and thickness up to six -months post-treatment when delivered at a high dose, accompanied by a partial correction of the localisation of Syntaxin 3, a partner protein of BBS10. Interestingly, a 5-fold lower dose of AAV8.RK.hBBS10 was not therapeutic, although the equivalent dose of an AAV8.RK carrying mouse *Bbs10* was efficacious. These findings support the hypothesis that due to a species difference the potency of the AAV8.RK.hBBS10 is potentially underestimated when assessed in *Bbs10* KO mice. In parallel to the work in mutants, we performed a long-term safety study to overexpress human *BBS10* under the RK promoter in wild-type mice. Up to six months post-injection, no significant detrimental effects on retinal function or retinal morphology were observed paving the way towards translation. Application for rare pediatric disease designation is currently underway.

P0352

Molecular and immunocytochemical characterization of an iPSC line derived from a patient with Retinitis Pigmentosa type 41 related to a mutation in the *PROM1* gene

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PROM1 gene encodes the protein Prominin-1 which plays a critical role in the morphogenesis of photoreceptors outer segments. The c.1354dupT mutation in *PROM1* causes a premature stop codon and it has been related with different Inherited Retinal Diseases (IRD) phenotypes. The aim of this study was to demonstrate the expression of pluripotency genes (endogenous reprogramming factors), the absence of reprogramming vectors (exogenous reprogramming factors) and the expression of pluripotency surface markers in the iPSC line [RP]-FiPSC1-Ep5F-10.

Our group generated the iPSC line [RP]-FiPSC1-Ep5F-10 from a patient with Retinitis Pigmentosa type 41 related to the mutation c.1354dupT in the *PROM1* gene. The iPSC colonies were stained with AP Blue Membrane Substrate Solution test. The c.1354dupT mutation was sequenced through Sanger sequencing. To evaluate the expression levels of endogenous pluripotency factors, we used gene-specific primer-based quantitative reverse transcription PCR (qRT-PCR) for SOX2, OCT3/4, KLF4, LMYC and LIN28. Additionally, to confirm the absence of reprogramming vectors, we conducted a dual assay: copy number qPCR using genomic DNA(gDNA) targeting a region common to all reprogramming vectors (within the EBNA1 gene), and vector-specific primer-based qRT-PCR using complementary DNA (cDNA) for each exogenous reprogramming factor introduced by the vectors. Compared with positive and negative control respectively. Then, immunocytochemistry with pluripotency-related antibodies was performed.

The generated iPSC lines tested positive for AP activity, exhibiting distinct blue staining. Besides, all of them displayed immunoreactivity for the nuclear markers (OCT4, Nanog, Sox2) and cell

membrane markers (SSEA-3, SSEA-4, Tra1-60, Tra1-80) associated with pluripotency. The relative mRNA expression of the endogenous reprograming factors (SOX2, OCT3/4, KLF4, LMYC, LIN28) were higher in the patient´s iPSC lines compared to the control. Furthermore, the relative mRNA expression of exogenous reprograming vectors was lower in the iPSC lines than in the control.

A new iPSC line was successfully generated, showing pluripotency and could be valuable for further elucidating why patients with the same mutation express different phenotypes. Future experiments should include gene-edited patient-derived iPSC due to its potential as disease modelling tools to elucidate this matter question.

P0353

Genetic repair of iPSC line derived from Cone-rod dystrophy patient due to mutation in the *PROM1* gene

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The *PROM1* gene encodes the protein Prominin-1 (CD133) which plays a critical role in the morphogenesis of photoreceptors outer segments. Mutations in the *PROM1* gene are related to different Inherited Retinal Dystrophy (IRD) phenotypes including Cone-rod dystrophy (CORD). The aim of this study was to genetically correct the mutation c.1354dupT located in the exon 13 of the *PROM1* gene in the iPSC line [CORD]-FiPSC1-Ep5F-2 derived from a CORD patient.

The most accurate CRISPR/Cas9 machinery was determined by the web tool for genome editing CRISPOR, Molbiotools, and the DNAStar Lasergene software. The *in silico* information was confirmed by *in vitro* essays using the U2OS cell line to select the most accurate gRNA. The iPSC line [CORD]-FiPSC1-Ep5F-2 was dissociated using Accutase and resuspended in mTeSR™ Plus medium supplemented with CloneR™2. A total of $1x10^6$ cells were electroporated with the selected gRNA (1,5µL at 1µL/µg), the repair oligonucleotide (15 µL at 20µM) and Cas9-protein v2 TrueCut™ (1,5µL at 5µL/µg) using the Neon Transfection System, with pulse conditions: 1,200 V; 30 Ms; 1 pulse. The electroporated cells were plated at a single cell density of 50 cells/cm^2 on Matrigel coated 6-well plates using mTeSR™ Plus medium supplemented with CloneR™2. The mTeSR™ Plus medium was refreshed daily until the colonies reached a sufficient size for manual picking. Genetic restauration was confirmed by flow cytometry using the anti-CD133 antibody conjugated to APC.

The quantification of the CD133-positive cell population in flow cytometry assays revealed no expression in the iPSC line derived from the CORD patient and restoration of expression in the gene-edited iPSC line. CD133 expression was also confirmed in healthy iPSC control line.

The genetic repair technique successfully recovered the capacity of the iPSC line [CORD]-FiPSC1-Ep5F-2 to encode Prominin-1. The characterization of gene-edited iPSC derivatives, including RPE, photoreceptors, and retinal organoids, would help to elucidate PROM1-related IRD phenotypic variability.

P0354

Characterization of an inducible AAV9-hTDP-43 mouse model for amyotrophic lateral sclerosis

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Intracellular aggregation and mislocalization of the TAR DNA binding protein TDP-43 in the human brain have been suggested as a hallmark of familial and sporadic amyotrophic lateral sclerosis (ALS). Furthermore, transgenic mouse models overexpressing human (h)TDP-43 display neurodegeneration associated with cognitive and motor deficits. However, these models often show a rapid disease progression and high mortality, limiting their use in research. Therefore, we aimed to establish a novel inducible ALS mouse model with a milder disease progression. To achieve this, we stereotactically injected 12-weeks old male C57BL/6 mice bilaterally into the motor cortex with an adeno-associated virus encoding hTDP-43 (AAV9-hTDP43). At 3-, 6-, and 9-months post-injection, mice were behaviorally tested for motor deficits, followed by histological analyses of markers for neurodegeneration in the brain. Histological evaluation revealed an approximately 6-fold increase in hTDP-43 levels in the motor cortex, with the expression levels remaining in a similar range throughout the entire observation period. hTDP-43 was mostly expressed in NeuN- and Ctip2-positive neurons. Compared to AAV9-empty-vector injected controls, the hTDP-43 expressing mice displayed significantly increased astrocytosis at 3- and 6-months post-injection, and a significantly reduced size of the motor cortex at 9-months post-injection, suggesting persistent neuroinflammation. Furthermore, hTDP-43 mice showed a decreased performance during the wire hanging test already ~ 1.5 months after injection, and the latency to fall was even further reduced from 6-months post-injection onwards, indicating reduced motor strength. Moreover, overexpression of hTDP-43 led to the onset of abnormal hind limb clasping behavior at 6-months post-injection, with the symptoms progressively worsening thereafter. Taken together, our data suggest that the inducible AAV9-hTDP-43 model exhibits a more moderate disease progression compared to classical transgenic models, while still maintaining an ALS-like phenotype. Therefore, this inducible model may be useful for pre-clinical studies investigating the mechanisms underlying ALS.

P0355

Transplantation of human stem cell-derived cone photoreceptors stimulates inner retinal neuron remodelling and improves visual function in the *Aipl1*$^{-/-}$ mouse model of end-stage Leber Congenital Amaurosis

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The retina is a highly organised, layered neural structure. The correct formation of the first synapse between photoreceptors, bipolar cells (BCs), and horizontal cells (HCs) is essential for light processing. During retinal degeneration, defects in photoreceptors or their support cells lead to photoreceptor death, a major cause of untreatable sight loss. Notably, the inner retinal neurons remain mostly intact, presenting an opportunity for visual restoration through photoreceptor transplantation.

We have previously demonstrated the restoration of retinal and visual function after transplantation of human pluripotent stem cell (hPSC)-derived cone photoreceptors (hCones) in the *Rd1* model of rod-cone dystrophy. To test the breadth of application for photoreceptor replacement therapy, we sought to rescue the *Aipl1*$^{-/-}$ mouse model of Leber Congenital Amaurosis (LCA), which exhibits rapid and widespread photoreceptor degeneration, with near-complete loss by postnatal day (P)18. This rapid degeneration disrupts the formation of photoreceptor-BC synapses in development, making it a particularly severe condition for rescue.

After hCone transplantation, cone BCs in the recipient retina showed substantial plasticity, extending dendrites towards the transplanted photoreceptors. Pre-synaptic protein ribeye and post-synaptic glutamate receptor mGluR6 were re-expressed, indicating nascent synaptic connectivity, while Multielectrode Array (MEA) recordings showed significant rescue of light-evoked activity. Synaptic blockers reversibly eradicated all fast transient responses confirming they originated from glutamatergic transmission between hCones and BCs in the outer retina. Transplanted mice also demonstrated optomotor head-tracking behaviour, which was completely absent in untreated mice.

Together with our previous demonstration of rescue of the Rd1 mouse, these findings indicate that photoreceptor replacement therapy is a viable disease-agnostic strategy, even in severe cases of retinal degeneration where extensive remodelling has occurred.

P0356

A mouse model with a mutation close to the O829X mutation in otoferlin causing progressive hearing loss: implications for gene repair approaches

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The protein otoferlin is essential for hearing. Mutations in the gene encoding otoferlin, OTOF, cause autosomal recessive profound prelingual deafness, DFNB9. The protein consists of six to seven C2 domains and a phospholipid-binding FerA domain the function of which is unclear today. The “Spanish” mutation, a frequently found premature STOP at position 829 (p.Gln829Ter), lies within the FerA domain, and is currently targeted by gene editing approaches. To better understand the function of the FerA domain for hearing, we have generated a novel mouse line with a mutation in the FerA domain. Our new Otof-p.KL>M mouse line has a three-base pair deletion that results in the replacement of lysine 824 and leucine 825 by a methionine. This mutation is predicted to impact an alpha-helix in the FerA domain involved in phospholipid binding. Using auditory brainstem response (ABR) measurements, we observed that homozygous Otof-p. KL>M mice exhibit slightly reduced ABR wave amplitudes at young age and a significant age-related hearing loss. 12 month old Otof-p.KL>M mice showed hardly any response to low and high frequency sounds, while at 4kHz, thresholds were 90 dB sound pressure level (SPL). As

expected, distortion product otoacoustic emissions (DPOAE) measurements were not affected in homozygous Otof-p.KL>M mice compared to the wild type. Consistent with the DPOAE recordings, we found no difference in the number of outer hair cells at any age. However, in the inner hair cells, Otof-p.KL>M mice show an age-dependent loss of inner hair cells at both the apical and the basal turn of the organ of Corti. Taken together, our findings provide a first insight into the role of the otoferlin FerA in hearing and, in particular, in inner hair cell survival. In addition, our mouse line could represent a model for point mutations due to gene repair approaches targeting the OTOF Q829X mutation.

P0357

AAV capsid variant screen for diabetes research: Building a high-throughput compatible methodology for modification of human islet gene expression

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Loss of functional β-cell mass in pancreatic islets is a key factor in the etiology of diabetes mellitus, one of the most prevalent metabolic disorders. Therapeutic strategies developed in animal models and β-cell lines are frequently not translatable to humans, which has impeded the development of a cure to this date. Isolated human islets, the current experimental gold standard for islet research, pose experimental challenges due to striking variability in their size, cellular composition, function, and purity, as well as low viability and functionality in prolonged culture. Additionally, their genetic manipulation is difficult due to poor penetration efficiency of transduction reagents, including viral particles.

To address these challenges, we developed a scalable adeno-associated virus (AAV) mediated transduction methodology in human islet microtissues. Through optimized dissociation and scaffold-free high-throughput reaggregation of primary islet cells in the presence of AAVs, we achieved high transduction efficiencies and organotypic islet architecture and function for over 28 days in culture. We chose AAVs for islet gene modification based on their efficacy and safety profile in preclinical and clinical studies. To identify the AAV capsid variant with the best natural affinity to transduce islets, we compared the tropism of nine AAV capsid variants encoding eGFP. Using 3D confocal microscopy and automated image analysis, we quantified viral transduction efficiency, transgene expression, and potential negative impacts on islet microtissue function and viability. Among the screened capsid variants, AAV2.7m8 exhibited the highest transduction efficiency and transgene expression with minimal effect on insulin secretion, insulin content and ATP levels. However, it strongly reduced the proliferative capacity of islets. AAV9 achieved lower transduction efficiencies and displayed more moderate gene expression compared to AAV2.7m8, but preserved islet proliferative capacity better.

Using AAV2.7m8, we knocked down dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), a previously characterized target known to induce islet cell proliferation. Harmine, a potent DYRK1A inhibitor, served as positive control. Following AAV transduction or harmine treatment and EdU incorporation, islet microtissues were stained in 3D for DAPI (nuclear marker), NKX6.1 (β-cell marker), and EdU (proliferation marker), imaged on a high-content confocal microscope, and analyzed for total cell count, β-cell count, fraction of proliferating β- and non-β-cells, per islet microtissue, using an automated 3D analysis pipeline. DYRK1A knockdown via

AAV2.7m8 increased total proliferation within human islet microtissues but was unable to induce proliferation in β-cells, while harmine was effective in inducing proliferation in both β-and non- β-cells.

In summary, we developed a scalable platform for AAV-mediated gene expression modification in human islets, achieving high transduction efficiency while maintaining stable islet function. This platform thus enables the study of putative therapeutic targets directly in human islets, potentially contributing to the future development of effective diabetes drugs.

P0358

Validating a neuroimaging biomarker for evaluating brain circuit function in angelman syndrome through gene therapy

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Angelman syndrome (AS) is a complex neurodevelopmental disorder caused by the loss-of-function of *UBE3A* in neurons. This condition manifests with profound cognitive impairment, behavioural alterations, seizures, and motor deficits. Currently, effective therapies for AS remain elusive, presenting considerable challenges for patients and caregivers. Despite ongoing investigations into various interventions, the accurate evaluation of their therapeutic efficacy is hindered by the absence of quantitative, reliable, and unbiased biomarkers for tracking disease progression and treatment response. We aim to bridge this gap by employing Intrinsic Optical Signal (IOS) imaging, a non-invasive neuroimaging technique of high translational value, to explore whether visually evoked responses can serve as a functional biomarker for monitoring the severity and progression of this disorder. Longitudinal monitoring of visual IOS (V-IOS) in Ube3a-deficient mice modelling AS (B6.129S7-Ube3atm1Alb/J) and control animals at postnatal day (P) 45 and P90 revealed a significant enhancement in the amplitude of V-IOS responses elicited by contralateral eye stimulation in HET mice compared to WT control mice. This demonstrates the ability of V-IOS to differentiate mutant subjects and controls in the animal model of AS. Furthermore, we investigated the capability of V-IOS biomarker to predict disease severity. We found a correlation between V-IOS amplitude and behavioral performance in marble-burying and rotarod tests. Specifically, higher absolute amplitudes of V-IOS were associated with poorer performance in these behavioral tasks. Finally, we assessed the rescue effects of gene therapy on V-IOS measures of HET mice. Preliminary results indicate that the re-expression of the UBE3A protein in the brain of AS mice reverses the alteration in V-IOS amplitude. Taken together, these findings suggest that V-IOS could serve as a promising biomarker of brain function for AS.

P0359

Upscaling And Differentiating Human Induced Pluripotent Stem Cell Spheroids In 3D J-E Sam1

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The efficacy of current treatments targeting neurological diseases are affected by their inability to cross the blood-brain barrier. Innovative study models to circumvent this is critical. Human induced pluripotent stem cells (hiPSCs) are capable of indefinite cultivation and differentiation into any cell, including traditionally inaccessible cells critical for brain barrier function like astrocytes. hiPSCs thus provide a path for developing novel therapeutic products. The challenge in implementation is the critical need for billions of high-quality cells. Thus, we aimed to develop methods for large-scale hiPSC culture for subsequent differentiation to astrocytes in 3D. To scale-up hiPSC culture we have utilized the SCINUS Cell Expansion System (SCES); a semi-automated bioreactor offering lower costs, labor and shear stress than other agitating systems. Upscaled hiPSCs in suspension were then tested for their capacity to differentiate into astrocytes.

hiPSCs were cultured as self-assembled spheroids in 6-well plates at a density of 1.5x105 cells/ml, maintained on an orbital shaker agitating at 70 rpm. Scaling up required determining critical culture parameters by imposing variations in refreshing regimes, seeding densities and agitation regimes. Morphological analysis, cell viability, and fold change, were used to evaluate favorable parameter combinations. Spheroid homogeneity and differentiation capacity was tested by inducing differentiation to astrocytes. hiPSC-derived neural progenitor cells (NPCs) were characterized using Nestin, Pax6 and FOXP2 staining.

The balance in cell density, medium refreshment and agitation regimes were improved during this study to maintain the growth and proliferation of hiPSC spheroids (Figure 1). Rocker speeds upwards of 400˚ /s were critical to control spheroid size ensuring nutrient availability until harvest. Results demonstrate robust cells with three SCES passages for the first time via Accutase dissociation. Spheroids were also successfully differentiated to astrocytes in suspension (Figure 2). hiPSC-derived NPC spheroid stains showed homogenous marker expression and indicated that spheroid size was adequate to maintain nutrient availability.

Overall, we were able develop a method to upscale hiPSC spheroid culture in a large-scale bioreactor for subsequent differentiation. This work offers promising methods for generating large quantities of cells needed for neurological therapeutic applications.

P0360

Physiologically triggered synthetic gene circuits in macrophages

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In the treatment of various diseases, the administration of cells for therapeutic purposes has become a powerful procedure in modern medicine. The generation of such cell therapy products

include *in vitro* manipulation to achieve specific gene expression pattern. Current research efforts are aimed at improving the specificity by tight temporal and spatial regulation of transgene expression. We aimed at developing cells that activate transgene expression only upon infections. To this end, we exploit type 1 interferon, a biomarker of infection, to activate a synthetic expression system by rewiring the cellular IFN signalling pathway.

To apply these features to the synthetic system, the doxycycline-controlled synthetic transactivator (tTA, TET-off) was first introduced into the locus of the interferon-stimulated gene Mx2 as a redirection module by monoallelic exchange. In a subsequent step, a Tet promoter-controlled cassette with a GOI and reporter genes was introduced into the previously characterized collagen type I (Col1a1) or the TIGRE locus, thereby obtaining predictable expression properties. For both integration sites, we demonstrate sensitive, dose-dependent reporter gene expression (luciferase, GFP) in different cell types (fibroblasts and macrophages) which strictly depended on type I interferon (IFN-β) and which was completely suppressed by addition of doxycycline. Implementation of the inducible caspase 9 as a GOI resulted in cells in which apoptosis was triggered by physiologically relevant concentrations of IFN-β. To ensure efficient genetic modification in therapeutic relevant cell types, we implemented the synthetic circuit in mouse embryonic stem cells since they provide high clonal expandability, scalability and *in vitro* differentiation capacity into various cell types of choice (e.g. macrophages, fibroblasts). We *in vitro* differentiated the modified ES cells to macrophages and fibroblasts. Importantly, the tight IFN dependent expression control could be confirmed in these cells.

We suggest that such self-regulating sensor/effector cells enable the controlled production of therapeutic agents during bacterial or viral infections. In this way, a self-adjustment of the level and temporal course of transgene expression could be achieved specifically at the site of infection.

P0361

Modelling X-linked myotubular myopathy in 3D human engineered skeletal muscles: prospects for gene therapies

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X-linked myotubular myopathy (XLMTM, also known as X-linked centronuclear myopathy, XLCNM) is a congenital myopathy, caused by mutations in the *MTM1* gene, characterised by muscle weakness, hypotonia and atrophy. Histologically, patient biopsies show reduced fibre size, and mispositioning of organelles, including nuclei and mitochondria. *MTM1* encodes for the ubiquitously expressed phosphatase myotubularin which is involved in membrane and organelle trafficking by regulating phosphoinositides. The pathogenic role of myotubularin in XLMTM is still not fully elucidated and to date no cure exists. Current XLMTM *in vitro* models either rely on invasive muscle biopsies, do not capture the complexity of the disease or of human skeletal muscle or often don't accommodate long term culture. *In vivo* XLMTM models are associated with ethical and financial burden, and have not optimally predicted safety concerns, halting a recent

clinical trial for XLMTM-gene therapy which led to fatalities caused by liver toxicity unforeseen in pre-clinical investigations. This underlines the need for stable, reproducible and human disease models to test treatment efficiency and predict outcomes.

We therefore generated a human advanced disease model of XLMTM from human immortalised and induced pluripotent stem cell (iPSC)-derived myoblasts that harnesses the power of bioengineering tissues to generate a *quasi-vivo* 3D-muscle model from healthy and XLMTM patient derived cells. This model facilitates the investigation of tissue architecture, function and muscle development in health and disease. When generating engineered muscles from human immortalised myoblasts, both patient and control samples showed myofiber alignment along the main axis of tension, and skeletal myogenic differentiation and maturation of myotubes. The myofiber diameter was found to be reduced in *MTM1*-mutant muscles, recapitulating the fibre hypotrophy observed in XLMTM patients.

To further investigate early-onset, disease-associated phenotypes an XLMTM iPSC line and an isogenic corrected control were generated and differentiated into the skeletal myogenic lineage. Similarly to the findings in immortalised muscle, patient derived 3D engineered muscles were found to have reduced fibre sizes compared to the isogenic control, indicating that that myofiber diameter is likely associated to early disease relevant changes in myogenesis. We are now harnessing this 3D-engineered muscle platform to investigate organelle mispositioning, including nuclear and mitochondrial dynamics to shed light on other pathological features observed in XLMTM patient muscles. Moreover, this platform will be used to assess efficiency, toxicity and cell-and tissue-specificity of genetic therapies with high fidelity in a human-specific environment.

P0362

High Efficiency, 2D, Embryoid Body-free, Feeder-Free, Xenofree and Fully Defined iPSCs Derived NK cell Production for Immunotherapy

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Natural Killer (NK) cells have been recognized as the first line immunosurveillance of our immune system that can employ lytic machinery to remove abnormal cells without pre-stimulation. Their distinct innate characteristics, shorter lifespan, and better safety profile compared to T-cells make them ideal for 'off-the-shelf' immunotherapy. Recently, allogeneic NK cell-based immunotherapy has emerged as a promising platform for cancer treatment when combined with chimeric antigen receptors (CAR). However, significant scientific challenges persist in current differentiation protocols for generating functional NK cells from human induced pluripotent stem (iPS) cells, related to efficiency, reproducibility, and scalability and caused by the use of embryoid bodies (EB), the use of feeder cells, and the use of animal or human-derived products (the latter also introduces the risk of contamination). Hence, there is a need to develop new methods for deriving functional allogeneic NK cells for the full potential iPS-derived NK cells to be reached as off-the-shelf therapies. To address these challenges, we have established a technically simplified iPS cell differentiation system for the efficient and reproducible production of NK cells that is fully defined, xeno-free, feeder cell-free, and does not rely on EB differentiation, thereby reducing major contributors to stochastic variability between in vitro derived preparations and significantly simplifying scalability. We demonstrate the importance of creating hemogenic niches that mimic

definitive hematopoietic development for the generation of mature functional NK cells. Furthermore, we demonstrate the cytotoxic killing activity of IPS-NK cells is comparable to that of NK cells derived from human peripheral blood. Our data towards developing a highly translational iPS-derived NK cell differentiation system is a promising and important step towards advancing NK-based immunotherapies.

P0363

Modeling ALSP Patient-Derived iPSC-Based Disease Modeling and CRISPR/Cas9 Isogenic lines in Microglia and Neurons : to investigate ALSP phatomechanisms

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Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) is a rare autosomal dominant disorder affecting the white matter of the brain, leading to cognitive impairment, caused by mutations in the Colony stimulating factor 1 receptor (CSF1R) gene. CSF1R affects brain cells such as microglia and neurons, playing a crucial role particularly in the proliferation and survival of microglia and the differentiation of neural progenitor cells. Previous studies have explored microglial dyshomeostasis using CSF1R haploinsufficient animal models; however, the exact mechanism between ALSP and the CSF1R gene remains elusive. As the importance of studying mechanisms using induced pluripotent stem cell (iPSC) technology grows, applying this approach to understanding ALSP pathology is crucial, as it allows representation of patient-specific mutations and differentiation into disease-specific cell types. Therefore, we aim to investigate the pathomechanism of ALSP disease and CSF1R mutations through patient-specific disease modeling using iPSC-derived microglia and neurons, comparing them with isogenic lines generated by CRISPR/Cas9 system.

To examine the impact of CSF1R mutations on microglial and neuronal dysfunction in ALSP, we established ALSP disease models by generating iPSC lines from 3 patients and creating isogenic lines using CRISPR/Cas9, which were then differentiated into microglia and neurons. These ALSP-iPSC lines were derived from peripheral blood mononuclear Cells (PBMCs) of the 3 ALSP patients who harbored each c.1765G>A, c.2546_2548delTCT, c.2345G>A CSF1R mutations through transduction of reprogramming factors using Sendai virus. For isogenic lines (ALSP-iPSC^{iso}), ALSP-iPSCs were edited using CRISPR/Cas9 to revert the CSF1R mutation to a normal genotype and were sorted by fluorescence-activated single-cell sorting (FACS). Furthermore, each ALSP-iPSC lines and ALSP-iPSC^{iso}lines were differentiated into microglia-like cells and neuronal cells which were obtained through specific differentiation stages from iPSCs: hematopoietic progenitor cells (HPCs) for microglia-like cells and neural progenitor cells (NPCs) for neuronal cells.

To validate our patient-derived iPSC-based ALSP disease model, we verified that each cell line derived during iPSC generation and differentiation into microglia-like cells and neuronal cells exhibits characteristic features specific to their respective cell types. We confirmed pluripotency maintenance, normal karyotype, and accurate reflection of each patient's CSF1R mutation type in

all generated iPSC lines Additionally, successful correction of CSF1R mutations in ALSP-iPSC^{iso} was confirmed through Sanger sequencing, without any observed off-target effects. To further identify characterization of iPSC derived differentiated cell types, immunofluorescence or flow cytometry was used with each representative marker.

In this study, we generated patient-derived iPSC-based ALSP disease model via differentiation into microglia-like cells and neuronal cells, including isogenic lines by CRISPR/Cas9. Confirmed ALSP-iPSC disease model platform enables us to investigate the role of CSF1R mutations in ALSP. Further study is on the way to compare the transcriptomes of microglia-like cells and neuronal cells derived from ALSP-iPSC lines and ALSP-iPSC^{iso} using RNA sequencing. By validating whether the differentially expressed genes (DEGs) identified through RNA sequencing are expressed in our created in vitro ALSP disease model, and assessing their impact on microglial phagocytosis, inflammation, or neuronal function, we aim to uncover novel mechanisms through which CSF1R mutation contributes to ALSP pathogenesis.

P0364

Revolutionary RNA delivery technology enables safe and efficient gene transfer in organoids, in vivo models, IPSCS, and stem cells

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The rapid evolution of cell and gene therapy calls for innovative gene delivery systems, able to respond effectively to the various strategies that can be envisaged, from gene expression to gene correction/replacement. Although DNA-based therapies using integrative lentiviral vectors and AAV have become widely available on the market, RNA-based therapies offer greater versatility and cover a wide range of applications with minimal risks. These therapies are particularly well suited to treat or prevent a wide variety of diseases. They are designed for applications where transient expression is safer, such as stimulating a cellular process, modifying a genetic sequence or directing cells towards a specific pathway. Depending on the disease, the delivered RNAs are designed to align with the selected therapeutic strategy, whether gene editing, regenerative medicine or immuno-oncology. RNA optimisation varies according to the target cells, the *ex vivo* or *in vivo* approach required, the genes of interest, and the intended level and duration of expression.

Organoids are three-dimensional stem cell-derived cell culture systems capable of mimicking the architecture and function of real organs. These miniature organ-like structures are invaluable tools for biomedical research thanks to their ability to closely mimic the *in vivo* environment. Despite persistent difficulties and the unmet need for new therapeutic strategies, organoids have emerged as a promising animal-free tool, revealing patient heterogeneity and enabling in-depth preclinical therapeutic screening. These tools are revolutionising drug discovery, personalised medicine, regenerative medicine, and offering new strategies for genetic modification. Flash BioSolutions has developed a revolutionary RNA technology, called FlashRNA®, to transfer RNAs safely and efficiently thanks to its unique features. It can deliver multiple types of RNAs in a highly specific way via a patented encapsulation method. Thanks to a robust multilayer particle, the RNAs are protected from degradation, delivered very efficiently into the cytoplasm of any cell types and immediately available for a rapid translation into proteins, without any risk of cell damage or phenotype alteration. FlashRNA®, which enables precise and efficient control of gene

expression and modulation, has demonstrated its ability to transfer RNA and lead to efficient protein expression in organoids to be further used for various studies. FlashRNA® is thus proving useful for improving the accuracy of disease modelling, drug development studies and screening, and even the development of personalised medicine. FlashRNA® has also demonstrated the ability to edit the genome of iPSCs, without inducing toxicity or adverse effects on their differentiation capacity. This efficient and safe delivery paves the way for its further use for specific applications such as genome editing on pluripotent stem cells and therapy.

In summary, FlashRNA® represents a breakthrough for cell and gene therapy, offering a powerful, safe and flexible delivery system for organoids, iPSCs and *in vivo* approaches. Its ability to induce efficient and precise genetic modifications positions it as a leading technology for advancing therapeutic strategies, with promising implications for all therapeutic areas. Furthermore, FlashRNA®'s existing cGMP-compliant production capabilities and rapid customisation allow to be promptly adapted to a variety of therapeutic needs.

P0365

Limited expansion of immortalized human hematopoietic stem and progenitor cells

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Hematopoietic stem and progenitor cells (HSPCs) represent the apex of the hematopoietic hierarchy, essential for the generation of all blood and immune cells. This unique positioning renders HSPCs ideal candidates for gene therapy in treating monogenic blood disorders. Additionally, the multipotent nature of HSPCs allows for their differentiation into diverse lineages, serving as an invaluable model for investigating the biological processes of differentiation and the pathological mechanisms underlying various diseases. Recent advancements underscore this potential, as evidenced by studies using the immortalized erythroid progenitor cell line HUDEP-2 to identify novel targets for activating fetal hemoglobin. Notably, these efforts have led to the landmark development of the first CRISPR-based product for gene therapy in sickle cell disease (SCD), marking a significant milestone in therapeutic innovation. However, immortalization of human HSPCs remains a challenge. In this study, we isolated primitive HSCs and developed a culture system supporting the maintenance of its stemness during the immortalization process, which involves overexpression of hTERT and a transcription factor associated with the stemness. Selected immortalized cells demonstrated robust proliferation for up to 70 days, colony-forming potential, and a multilineage potential with a strong bias towards macrophages. After 70 days, the proliferation rates decreased, accompanied by gradual cell death. This research underscores the potential of immortalizing HSPCs and emphasizes the need for a deeper understanding of HSC biology to fully achieve success in developing immortalized HSPCs.

P0366

AAV-intein gene therapy is effective in a novel large animal model of Stargardt disease

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Stargardt disease type 1 (STGD1) is the most common form of inherited macular degeneration, for which no therapeutic options are available. STGD1 is due to bi-allelic loss-of-function mutations in *ABCA4* which has a large coding sequence that does not fit into adeno-associated viral (AAV) vectors, the most suitable vectors for retinal gene therapy. We have recently developed a gene therapy approach for STGD1 which relies on the use of two AAV vectors each encoding for one of the two halves of the ABCA4 protein flanked by split-inteins, that catalyze seamless joining of the two ABCA4 half proteins following AAV administration. Based on the finding that subretinal administration of AAV-*ABCA4*-intein vectors results in precise reconstitution of the full-length ABCA4 protein and in therapeutic efficacy in *Abca4-/-* mice, we set to evaluate the therapeutic efficacy of AAV-*ABCA4*-intein vectors in relevant large animal models.

Given the similarity between the swine and human retina, we have generated ABCA4 knock-out (KO) pigs, by CRISPR/Cas9-mediated ABCA4 editing followed by somatic nuclear transfer. While KO pigs do not show obvious signs of photoreceptor degeneration up to 15 months of age, they accumulate lipofuscin in the retinal pigmented epithelium similarly to Abca4-deficient mice and STGD1 patients. Subretinal delivery of AAV-*ABCA4* intein vectors in ABCA4 KO pigs results in efficient reconstitution of the ABCA4 protein and reduction in lipofuscin accumulation at 2 months post-injection, supporting further clinical translation of this platform for gene therapy of STGD1.

P0367

Characterization and therapeutic potency evaluation of SCA3/MJD iPSC-derived neuroepithelial stem cells silenced for mutant *ATXN3*

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Machado-Joseph Disease (MJD) is a severe neurodegenerative disease caused by an abnormal expansion of the polyglutamine tract in the mutant ataxin-3 protein, whose aggregation triggers neuronal death in several brain regions including the cerebellum. Recovery of MJD patients in advanced stages of the disease would require an efficient neuronal replacement of the dead neurons. Recently, we demonstrated that human induced pluripotent stem cell-derived neuroepithelial stem cells (iPSC-derived NESCs) derived neurons and bystander effects are maintained up to 6 months after cerebellar transplantation (Mendonça et al 2024). Hereupon, this work aimed at generating, characterizing, and evaluating the therapeutic potential of MJD iPSC-derived NESCs silenced for the mutant *ATXN3* (mutant ATXN3-silenced NESCs) for cell-based personalized therapies.

The previously established and characterized MJD iPSC-derived NESCs (Mendonça et al 2024) were silenced for the mutant *ATXN3* with shRNAs against the mutant *ATXN3* mRNA delivered by lentivirus also encoding for puromycin resistance gene, for cell selection, and with or without copGFP reporter gene. The resulting silenced cells showed reduced mutant *ATXN3* mRNA and protein levels by 33-70% and 50-60%, respectively (depending on the construct), and no significant impact on the wild-type ataxin-3 protein levels was observed. Mutant ATXN3-silenced NESCs stable lines expressing Nestin and capable of differentiating into S100B-positive glia and MAP2-positive neurons were established. Functional neurons responding to KCl and not histamine stimulus was observed through single-cell calcium imaging. Moreover, no significant differences were observed between mutant *ATXN3*-silenced NESCs and MJD NESCs treated with shRNA CNT for the number of functional neurons and excitatory ($PSD95^+$ and $VGluT1^+$) and inhibitory ($VGAT^+$ and $Gephyrin^+$) synapses. Together these results showed that the MJD mutation could be silenced in NESCs without compromising their multipotency and ability to originate functional neurons. The mutant *ATXN3*-silenced NESCs expressing no reporter gene (CoP GFP) showed better survival after being transplanted by stereotaxic injection into the brain of immunocompromised (NOD/SCID) mice. Then, mutant ATXN3-silenced NESCs expressing no reporter gene and Control NESCs were transplanted into the cerebellum of immunosuppressed MJD transgenic mice resulting in motor coordination impairments amelioration. Mutant ATXN3-silenced NESCs and Control NESCs were also transplanted into the striatum of a lentiviral-induced MJD NOD/SCID mouse model. NOD/SCID mice developed MJD neuropathological hallmarks, such as mutant ataxin-3 aggregates, neuroinflammation, and neuronal loss, through the lentiviral-mediated expression of the human mutant ataxin-3 protein in the striatum. Both Control and mutant ATXN3-silenced NESCs differentiated into glia and neurons. Graft-derived human neurons with excitatory and inhibitory synapses in the host brain were detected 2 months upon cell transplantation. Moreover, no major signs of immune rejection or graft overproliferation were observed, attesting to the safety of the cell transplantation. Regarding the MJD-associated neuropathology, a significant reduction in the size of the mutant ataxin-3 protein inclusions was observed for both cell lines; other neuropathological markers are presently under evaluation.

Although further investigation is needed, these results suggest that Control and mutant ATXN3-silenced NESCs have therapeutic potential providing, to some extent, neuroprotection from the MJD-associated neuropathology.

P0368

Development and characterization of iPSC-derived regulatory T cells – Towards an allogenic cell therapy for the treatment of autoimmunity and inflammatory conditions

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Regulatory T cells (Tregs) play a pivotal role in maintaining immune homeostasis and preventing autoimmunity. Treg-based cell therapies have already shown efficient modulation of immune responses in several clinical settings. However, the complexity and the cost of manufacturing autologous cell products restricts their implementation to certain patient populations with the highest unmet need. Induced pluripotent stem cells (iPSCs) have emerged as suitable starting material for generating diverse cell types, including Tregs. This abstract highlights our efforts in deriving iPSC-based Tregs and characterizing their functionality for potential therapeutic applications.

Through rigorous optimization, we developed a differentiation process that recapitulates the natural development of Tregs. This included in vitro exposure of iPSCs to conditions that promoted the expression of factors associated with Treg lineage commitment, while enhancing cell yield and purity, and minimizing cell death.

Based on flow cytometry analysis, live populations of ~60% FOXP3+/CD25+/CD127-/CD4+/CD3+/TCRαβ+ cells were derived with a total expansion fold of 1 iPSC to 6,000 Tregs. The phenotype of iPSC-Tregs included expression of additional markers associated with Treg identity and function, including Helios, CD39, and CTLA-4. Purified cells positive for these markers had highly demethylated FOXP3 TSDR loci as determined by epigenetic analysis, thus demonstrating a natural Treg (nTreg) vs. induced Treg (iTreg) identity. iPSC-Tregs were capable of becoming activated upon exposure to appropriate stimuli, supported by upregulation of CD69, GARP and CD137 markers. Importantly, the expression of pro-inflammatory cytokines was equivalent to the levels observed in primary nTregs. Functionality testing demonstrated suppressive capacity towards effector T cell proliferation and inflammatory cytokine production in a dose-dependent manner. Additionally, iPSC-Tregs demonstrated phenotype stability when challenged under Th-1 and Th-17 skewing cell culture conditions.

Our results demonstrate the successful derivation of functional iPSC-Tregs exhibiting potent suppressive activity. Flow cytometry and epigenetic analyses confirm the identity of the Treg lineage, reinforcing the fidelity of our differentiation protocol. This study provides a robust platform for the derivation and functional characterization of iPSC-Tregs, paving the path towards their potential application in cell therapy against autoimmune, metabolic, and other inflammatory diseases.

P0369

Promoting the formation of a pseudo-optic nerve from human retinal organoids on aligned nanofibre scaffolds

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Retinal degenerations are the leading cause of untreatable sight-loss in industrialised countries. Retinal development and disease are studied in a wide variety of small and large animal models and therapeutics are typically developed with use of these same animal models. The retina comprises three layers of neurons; the light-sensing photoreceptor layer, the inner retinal layer and the retinal ganglion cell (RGC) layer, which sends visual information via axonal projections through the optic nerve to the brain.

Stem cell-derived laboratory-grown tissues, or “organoids”, offer extraordinary potential to study human ocular disease and test new treatments, including gene and cell therapy, drug screening and substantially reduce the requirement for animal studies. Human stem cell-derived “retinal organoids” (hROs) are spontaneously forming tissues. In many ways, the resulting hROs are remarkably similar to human retinae, presenting with well-formed photoreceptor layers and all main classes of retinal neurons, but they are imperfect, failing to replicate many important aspects of retinal structure, connectivity and function.

A common phenomenon that occurs before full maturation of photoreceptors, is the breakdown of inner retinal structure and loss of RGCs, most likely because they lack a target &/or nutrient access. Bioengineered scaffolds can mimic many structural features of tissues; here we have developed a custom-designed hRO culture platform using soft lithography techniques for organoid seeding onto nano-scale uniaxially aligned fibres to create a scaffold for RGC axons to grow along. These have been combined with hROs with very encouraging preliminary results, where RGCs send axons along the directed fibre creating a pseudo-optic nerve, and, importantly, survive in large numbers for extended periods in culture. We are currently seeking to fully develop this platform to guide the growth of an optic tract in a developmentally relevant and reproducible manner. We will be characterising control hROs and platform grown hROs for organoid size, RGC viability, connectivity and ability to elicit a robust electrophysiological response to light. Addition of a nanofibre scaffold is anticipated to yield better organised, more mature structures that can support improved connectivity such that these can be used to model light response behaviour in human-derived retinal tissue to study inherited photoreceptor/RGC diseases.

P0371

Disease exacerbation in MYOrganoids derived from Duchenne Muscular Dystrophy iPSC reveals limitations of microdystrophin therapeutic efficacy

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Duchenne muscular dystrophy (DMD) is a lethal muscle-wasting disease caused by the absence of Dystrophin, a protein essential to preserve muscle integrity continuously challenged by contractions. Gene therapy utilizing adeno-associated virus (AAV) to deliver truncated forms of dystrophin (microDys) is currently the most promising therapeutic approach. However, the therapeutic outcome in treated patients has not been as successful as anticipated by animal studies, underscoring the need for improved and high-throughput models for fast and accurate prediction of human response. Among the in vitro cellular models, organoid-like structures are becoming an appealing resource for disease modeling and replacement of animal models.Here, we describe the generation of MYOrganoids, an in vitro 3D muscle platform based on direct myogenic conversion of human induced pluripotent stem cells (iPSC) including fibroblasts to ensure proper muscle structure and function. We also exploited the secretory activity of fibroblasts to provide microenvironmental cues, essential for pathophysiological studies. Remarkably, MYOrganoids derived from DMD-iPSC including DMD fibroblasts, show exacerbated pathogenic hallmarks such as extracellular matrix remodeling, muscle force loss and fatiguability, across the different DMD iPSC cell lines employed, independently from the genetic background of the DMD fibroblasts used.

As proof of the suitability of our system for gene therapy screening, we employed AAV9-mediated µDys gene transfer in DMD-MYOrganoids. We showed that µDys delivery, partially improved muscle resistance but failed to significantly restore dystroglycan components at the membrane. Transcriptomic analysis confirmed an amelioration of mechano-stability and inflammatory hallmarks but, more importantly, revealed that only a partial correction of the DMD signature is achieved after microDys restoration. This evidence highlights the necessity to identify additional therapeutic targets and places our bioengineering approach at the forefront of exploring complementary strategies beyond gene therapy with the potential to accelerate the discovery of more effective therapeutics.

P0374

From iPSCs to mature cortical neurons: a novel protocol for high-efficiency differentiation

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The generation of cortical neurons from induced pluripotent stem cells (iPSCs) holds significant promise for both research and therapeutic applications. However, existing protocols often face limitations in scalability and preservation of neuronal properties through expansion and cryopreservation stages. We have developed a novel protocol to generate cortical neurons from induced pluripotent stem cells (iPSCs) with an enhanced yield and preservation of cortical attributes. This process involves an initial production of neural progenitor cells (NPCs), which can be frozen, thawed, and expanded up to five passages before maturation into cortical neurons. The protocol's robustness lies in its flexibility, allowing procedures to be paused and resumed without sacrificing cell yield or cortical identity.

Neurons generated via this protocol have been rigorously validated through comprehensive biomolecular and electrophysiological investigations. Neuronal markers were quantified at two maturation stages (days 28 and 42) using quantitative real-time PCR (qPCR), and compared to those from iPSCs and adult human cortex. The markers examined include: NANOG, SOX2, PSD95, SYN1, GAD1, GABRA2, SLC17A6, SLC17A7, B3TUB, MAP2, TBR1, CTIP2, NEUN AND

GFAP. qPCR validation showed a decrease in pluripotency markers NANOG and SOX2, with increased neuronal marker expression that further corroborated the cortical nature of the generated cells. Immunocytochemistry was utilized to assess cellular morphology and the localization of neuronal markers, providing further support for the necessary morphological differentiation and maturation of cortical character.

Furthermore, patch-clamp results found immature action potentials at day 28, indicating nascent neuronal functionality, which further matured to fully fledged action potentials by day 42, suggesting functional maturation over time. Comparatively, calcium imaging revealed no significant differences in calcium activity between stages, showing consistent functional development.

This novel protocol thus provides a reliably efficient method to produce high-yield, functionally matured cortical neurons from iPSCs. This novel protocol provides a valuable tool in neuroscience, creating a feasible iPSC-derived cortical neuron model for assessing neurotoxicity and potentially contributing to the development of patient-specific therapeutics.

P0375

Advances and challenges in generating new models for the rare genetic disease Mucolipidosis type II

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Mucolipidosis type II (ML II; MIM 252500) is an autosomal recessive lysosomal storage disease (LSD) of hydrolase trafficking for which there is still no treatment. It is multi-systemic, with prenatal or neonatal onset and fatal outcome in early childhood. ML II is caused by mutations in *GNPTAB* that prevent the production of an active N-acetylglucosamine (GlcNAc)-1-phosphotransferase. The most frequent one is the homozygous frameshift mutation c.3503_3504del [NM_024312.5(GNPTAB):c.3503_3504del (p.Leu1168fs)].

Our group has been exploring the potential of RNA therapies for ML II. However, to test them and help overcome the difficulty of translating this kind of approach to clinical practice, new cellular and animal models are necessary, ideally carrying the disease-causing allele and representing different cell types.

Therefore, we generated for the first time, patient-specific induced pluripotent stem cells (iPSCs) for the most frequent ML II-causing mutation, that can be further differentiated into relevant cell types. Besides, we are now optimizing the CRISPR-Cas9 knock-in (KI) to correct that mutation thus generating an isogenic line. For this, five sgRNAs and two single strand DNA donors (ssDO)

were already tested, using electroporation as delivery method of the ribonucleoprotein (RNP) complexes. We found two sgRNAs to be efficient in inducing the cleavage, but no homology directed repair (HDR) events were observed yet, maybe because these sgRNAs are located over 10 bp away from the mutation (maximum distance recommended). So, optimizations are needed, namely regarding concentrations and using different Cas.

In vivo, we are also using CRISPR/Cas9 KI to create a zebrafish MLII model with the orthologous *gnptab* mutation correspondent to the most frequent in humans, suited for preclinical testing of new therapies. A TG deletion in exon 19 was determined *in silico* as the corresponding orthologous mutation. One sgRNA and one ssDO were tested and microinjected at 1-cell stage embryos. By day 3 post fertilization, larvae were anesthesized and lysed for genotyping that showed more than 80% frameshift efficiency in F0. From the 98 larvae analysed, 3 had the delTG in more than 50% of editing events. We are now optimizing concentrations to increase efficiency as well as to grow zebrafish to adulthood and select the heterozygous that inherited the mutation.

In conclusion, we are demonstrating some of the difficulties of the CRISPR/Cas9 KI approach for generating disease models, namely finding PAMs in close proximity to editing site and efficient sgRNAs. HDR is much less efficient than non-homologous end joining, so more optimizations and higher number of samples/embryos are needed. Nevertheless, we expect to overcome these difficulties and obtain two independent but complementary and valuable platforms to model ML II and allow for high-throughput screens.

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P0376

Correction of neuropathological features of Gaucher disease in microglia-containing cerebral organoids

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Gaucher's disease is a lysosomal storage disorder caused by biallelic mutations in the *GBA1* gene. These mutations alter the folding and function of the enzyme beta-glucocerebrosidase (GCase), leading to the accumulation of its glycolipid substrate in the lysosomes. Macrophages are primarily affected, but progressive encephalopathy (nGD) is observed in some patients. The molecular and cellular mechanisms by which the enzymatic defect and lipid overload lead to neuronal loss and increased neuroinflammation are currently unknown. Enzyme replacement and substrate reduction therapies fail to control the neuropathology. Our long-term goal is to develop an *ex vivo* gene therapy by modifying hematopoietic stem cells. Once infused into patients who have received myeloablative treatment, these cells can cross the blood-brain barrier, colonize the

central nervous system, and differentiate into microglia. If necessary, they can secrete a therapeutic protein that can penetrate neural cells. To evaluate the efficacy of vectors and therapeutic proteins, we aimed to establish an *in vitro* model of the neuronal pathology. We derived cerebral organoids from pluripotent stem cells (iPSCs) from a healthy individual, the same cells treated with a GCase inhibitor (conduritol-β-epoxide, CBE), iPSCs from a patient with the neurological form of the disease (*GBA1*$^{L444P/L444P}$), and the same mutated cells re-expressing the wild-type GCase. We show that the process of cellular differentiation is not affected by the mutation or by the catalytic inhibition of the enzyme. We can produce organoids containing neurons, astrocytes, and microglia from normal or mutated cells, with similar numbers of neurons and microglial cells. However, we observe significant abnormalities in the structure of mitochondria and rough endoplasmic reticulum in the mutated organoids or those in which GCase has been inhibited. These abnormalities are largely corrected in the mutated cells re-expressing the wild-type form of the enzyme. We observe a significant presence of reactive astrocytes in organoids derived from mutated and CBE-treated cells, with the proportion of astrocytes increasing in the presence of microglia. Therefore, the decrease in GCase activity is sufficient to causes cytological abnormalities and astrocyte activation. Surprisingly, we find that the *GBA1*$^{L444P/L444P}$ gene results in numerous transcriptional deregulations, whereas GCase inhibition by CBE has almost no effect on the transcriptome. Importantly, re-expression of the wild-type protein reverses these transcriptional anomalies. These results suggest that the phenotypic abnormalities observed in patients with the neurological form of Gaucher's disease result not only from the low activity of the enzyme but also from the specific properties of the *L444P* mutated enzyme. They also suggest that the presence of the wild-type enzyme compensates for all the anomalies caused by the mutation, both cytological and transcriptional. The neuronopathic form of Gaucher's disease resulting from the *GBA1*$^{L444P/L444P}$ gene could therefore be corrected by providing the wild-type protein. This model incorporating microglia and neural cells will allow testing the delivery of the therapeutic protein via microglial cells transduced by lentiviral vectors.

P0377

Scalable and consistent Manufacturing of functional lung cells from iPSCs: Ncardia's advanced differentiation protocols for drug discovery and tissue engineering

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Induced pluripotent stem cells (iPSCs) offer unparalleled potential for drug discovery, disease modeling, and tissue engineering due to their ability to self-renew indefinitely and differentiate into any cell type. However, the differentiation of functional cells from iPSCs with consistent quality and at scale remains a significant challenge. Variability in iPSC differentiation, influenced by numerous biological factors, complicates standardization, leading to increased risk, cost, and development time. Leveraging over a decade of innovation, Ncardia has developed robust, scalable procedures for differentiating iPSCs into various cell lineages, including the complex endodermal lineage, thereby enabling the production of functional lung cells for multiple applications.

The development process begins with client collaboration to define the target cell quality profile, including parameters such as identity, purity, potency, and scale. Ncardia's expert team then conducts parallel testing of various protocols using scalable platforms to establish proof of

concept. This iterative process identifies critical process parameters (CPP), critical material attributes (CMA), and critical quality attributes (CQA), facilitating the selection of optimal protocols for scale-up development. Throughout this process, robust data collection and analysis enable predictive modeling, ensuring consistent and reproducible outcomes.

Ncardia successfully developed scalable differentiation protocols for four main human lung cell types: distal type II alveolar epithelial cells (AT2), proximal lung basal cells, endothelial cells, and stromal cells. The iPSC-derived AT2 cells exhibited characteristic morphology with secretory granules (lamellar bodies) and expressed specific markers (ABCA3, SPC), essential for pulmonary surfactant production. Proximal lung basal cells, crucial for epithelial repair and maintenance, demonstrated typical morphology and expressed TP63 and KRT5. Endothelial cells displayed elongated shapes, formed vessel-like structures, and expressed CD31 and CD144, with functional capabilities such as LDL uptake. Stromal cells showed expression of PDGFRβ and SMA, with assessed growth and contractility, confirming their role in structural support and immune regulation in lung tissue.

Ncardia's approach addresses the challenges of iPSC manufacturing by minimizing variability and enhancing scalability, quality, and consistency. By integrating deep stem cell biology expertise with scalable platforms and data-driven decision-making, Ncardia accelerates process development while reducing risks. The successful differentiation of lung cell types from iPSCs highlights Ncardia's capability to produce high-quality cells in big volumes for drug discovery and regenerative medicine. This comprehensive work highlights Ncardia as a premier partner for advancing therapeutic development through innovative iPSC technologies, offering customized, large-scale manufacturing solutions that enable significant scientific and clinical breakthroughs.

P0378

Enhanced retina co-culture with standardized RPE cells as Drug Development Tool for intraocular Cell and Gene Therapy Medicinal Products

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Novel intraocular drugs and particularly cell and gene therapies need robust and reliable models for development of efficient and safe Advanced Therapy Medicinal Products (ATMP). Available Drug Development Tools (DDT) are often of limited transferability and fraught with ethical concerns due to animal experiments in species with different retinal anatomy and functionality compared to humans. *Ex-vivo* organ culture can be an alternative, however, current retinal are mostly limited to a duration of 4-8d and of animal origin. Recently, we cultured human retina for 14d. Currently, we are improving the system by prolongation of culture duration to 28d and co-culturing retinae with induced pluripotent stem cell (iPSC)-derived retinal pigment epithelial (RPE) cells (iRPE). The co-culture with iRPE cells will standardise the model, improve retinal preservation, and offer testing of ATMPs like genetically modified RPE cells or iRPE cells from patients. Moreover, additional induction of oxidative stress will mimic avascular age-related macular degeneration (aAMD) and offer drug discovery in an *ex-vivo* disease model. Here, we present intermediate results of the co-culture of porcine retinae in direct contact with primary RPE cells for 21d. Porcine eyes were received from a slaughterhouse 5-8h *post-mortem* and RPE

cells isolated and grown until confluence under standard conditions (DMEM/F12, 10% FBS, 1% antibiotics and anti-fungicides). At around 3 weeks, retinae were isolated from porcine eyes and co-cultured with RPE cells in direct contact to photoreceptors in culture inserts at 21°C or 28°C, and Ames' medium supplemented with 5% FBS, 1% N2, and 1% B27. RPE cells and retinae were analysed at 1, 7, 14, and 21d. Morphology and tissue integrity was evaluated by brightfield microscopy of flatmounts and hemalum and eosin (HE)-stained cross-sections. Propidium-Iodide staining determined necrosis while cell viability was analysed by performing the LDH-Glo™ Cytotoxicity assay. Our current model slows down degeneration in the retina by hypothermic culture at 21°C. The low temperature however, significantly damaged RPE cells as seen microscopically, while 28°C showed an acceptable compromise of cell damage and retina preservation towards an overall benefit for the co-culture in terms of improved retinal integrity analysed in HE-stained cross-sections up to 21d; the novel medium supported concurrent cell and tissue preservation. Necrosis was significantly decreased at 28°C compared to 21°C-co-cultures (9.64±5.70 vs. 26.26±10.54 mean grey value, $p<0.0001$). Similarly, viability increased (lower luminescence signifies higher cell viability) in 28°C-co-cultures compared to 21°C (92'729±17'761 vs. 114'055±27'771 AU). Results demonstrate the establishment of culture conditions for RPE cells and retinal tissue in direct contact for 21d with reduced degeneration and preserved cell and tissue integrity. Ongoing experiments are transferring co-culture to human retinae with iRPE cells and a culture duration of 28d. The innovative organ culture model will offer drug discovery in an *ex-vivo* retinal model for one month. Human tissue and iRPE cells will guarantee reproducibility, high transferability to human patients and thus safety of tested ATMPs; the further development of the model into an aAMD model will additionally enable robust study of efficiency of future intraocular cell and gene therapies.

P0379

Human induced pluripotent stem cell-based disease modeling for gene therapy of Bietti's crystalline dystrophy

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Bietti's crystalline dystrophy (BCD) is a progressive chorioretinal degenerative disease caused by mutations in the *CYP4V2* gene, ultimately leading to vision loss and blindness in patients. Currently, there are no effective treatments for BCD due to the lack of suitable human-derived disease models for meaningful exploration of the disease mechanisms and potential therapeutic strategies. Gene therapy, utilizing genome editing technology, has emerged as a promising strategy to treat BCD. Although animal models have been employed for studying disease mechanisms and testing therapies, interspecies anatomical differences limit their e ffectiveness for this purpose. Recent advancements in human induced pluripotent stem cell (hiPSC) technology offer a valuable opportunity to generate disease-specific hiPSCs from BCD patients, facilitating the creation of disease-relevant two-dimensional (2D) and three-dimensional (3D) derivatives for BCD modeling *in vitro*. In this study, we utilized hiPSCs derived from BCD patients carrying *CYP4V2* mutations to model BCD *in vitro*. Our results have demonstrated that BCD-hiPSC-derived retinal pigment epithelium (RPE) cells and photoreceptor cells recapitulated BCD-associated disease phenotypes. In addition to 2D cell cultures, a 3D retinal organoid system for

BCD disease modeling has been created. Characterization of BCD-associated disease phenotypes showed that lipid droplet accumulation occurs in RPE cells, and both RPE and photoreceptor cells within the BCD-hiPSC-derived retinal organoids were prone to cell death. Furthermore, the efficacy of genome editing was evaluated using the prime editor (PE) to correct the *CYP4V2* mutations, followed by various BCD-hiPSC-based cellular and functional assays, which showed that PE corrected the *CYP4V2* mutations, reduced RPE cell degeneration, and restored the function of BCD-hiPSC-derived photoreceptor cells. These findings highlight the potential of genome editing as a treatment approach for BCD.

P0380

Design of experiments assisted optimization of induced Pluripotent Stem Cell (iPSC) directed differentiation toward airway progenitors

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Primary ciliary dyskinesia (PCD) is a rare genetic disease causing ciliary function impairment and bronchial mucus accumulation among other comorbidities. We assume that the development of an autologous cell and gene therapy using iPSC derived airway progenitors could restore mucociliary function of PCD patients.

Common bottlenecks regarding iPSC differentiation are the persistence of undifferentiated cells and the emergence of unwanted cell types. In a therapeutic context, it can cause uncontrolled cell proliferation or differentiation and reduce the therapy efficiency. iPSC differentiation into airway progenitors often leads to the coexistence of hepatic and pulmonary cell lineages. This phenomenon was documented by a single cell RNA sequencing analysis of iPSC-derived progenitors. The emergence of hepatic progenitors occurs between the definitive endoderm (DE) and the anterior foregut endoderm (AFE) stages. This key differentiation step relies on the inhibition of the TGF-β and BMP pathways. In our standard protocol (STD), it is performed by removing all cytokines from the differentiation medium. Alternatively, a double inhibition (DI) can be realized by using inhibitors of both pathways.

A comparison of those two protocols (STD vs DI) showed that the DI reduces pluripotency and liver markers expression, improving pulmonary marker expression. Nevertheless, DI leads to cell mortality. For the following experiments, we chose to optimize the DI protocol employing a design of experiments (DOE) approach. This statistical method allows to optimize a protocol by evaluating the influence of different variables upon several response factors. First, a screening design is applied to identify which variable have a significant influence on the response. Then an optimization design is applied on the critical variables to identify their optimum value, allowing the obtention of a target response. To assess the efficiency of the iPSC differentiation, the relative expression of the main airway progenitor biomarker NKX2-1 has been measured by RT-qPCR as well as the expression unwanted cell-types markers. The viability of the cells in the end of the differentiation process has also been assessed.

The main effects shown by the screening DOE were caused by three variables linked to the transition from DE to AFE stages. Significant effects linked to the last step of differentiation have

also been observed. The optimization DOE has been applied to the three variables linked to the DE toward AFE differentiation stage and allowed significant modeling of the expression of several genes. The best condition of the optimization design allows more than a 2-fold increase of the NKX2-1 expression and a reduced expression of liver markers compared to our STD protocol without compromising cell viability. The optimized protocol remains to be tested to confirm these results and to assess the capacity of these NKX2-1+ lung progenitors to differentiate into bronchial epithelium. Furthermore, a single cell RNA sequencing analysis will be performed to fully characterize the homogeneity of the iPSC-derived progenitors.

P0381

Long-term metabolic, phenotypic, and neuropathological characterization of the *Cyp27a1*$^{-/-}$ mouse model of cerebrotendinous xanthomatosis

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Cerebrotendinous xanthomatosis (CTX) is caused by mutations in *CYP27A1* gene, encoding the mitochondrial sterol 27-hydroxylase enzyme involved in the bile acid synthesis pathway. *CYP27A1* deficiency results in the reduction of bile acid production and a dramatic decrease in 27-hydroxycholesterol, whereas some toxic precursors accumulate in the blood and some organs, especially the eyes, tendons and brain. As a consequence, CTX patients present cataracts, tendon and brain xanthomas, premature atherosclerosis, and progressive neurological damage including mental retardation, dementia, ataxia, seizures, and peripheral neuropathy. It has been reported that *Cyp27a1*$^{-/-}$ mice present milder accumulation of toxic metabolites and virtually no symptoms of the disease. To fully characterize this model, a long-term evaluation of the *Cyp27a1*$^{-/-}$ mice was conducted. B6.129-*Cyp27a1*^{tm1Elt}/J mice were obtained from The Jackson Laboratory (Ref. 009106) and a full metabolic, phenotypic, histological and neuropathological characterization of the mouse model was performed up to 18 months of age. *Cyp27a1*$^{-/-}$ mice had hepatomegaly, increased expression of *Cyp7a1* and *Cyp3a11* and increased levels of plasma ALT compared to their WT littermates, as previously reported in the literature. After 6 months of age, *Cyp27a1*$^{-/-}$ mice also presented lower body weight gain and steatosis. Toxic bile acid precursors were elevated in circulation and in the brain of *Cyp27a1*$^{-/-}$ mice. Despite most behavioural tests showed no alterations, *Cyp27a1*$^{-/-}$ mice presented signs of ataxia, especially in females. To our knowledge, this is the first study showing measurable motor alterations in the mouse model of CTX, similar to those described in CTX patients.

P0382

Exploring the potential of long-term organotypic culture of the postmortem adult human retina for gene therapy development

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There is an increasing demand in research for three-dimensional human retina models. Here, we present an organotypic culture model that allows the postmortem adult human neural retina to survive in its full complexity for over 35 weeks.

Eyes of adult multi-organ donors without known ocular diseases were enucleated prior to cardiac arrest. Eyes from more than 150 donors were used in the study, ranging in age from 18 to 74 years. After dissection, organotypic cultures were prepared and placed on a polycarbonate membrane. The cultures were maintained for up to 245 days using a chemically defined serum-free medium. Light sensitivity of the cultures and spontaneous ganglion cell activity were evaluated using a multi-electrode array system. To demonstrate a significant application of the method, a subset of cultures was transduced with different viral vectors. Following fixation, the quality of the cultures was analyzed by immunohistochemistry and TUNEL assay.

The morphology of the cultures was remarkably well preserved with low variability between samples. All major cell types survived, and the integrity of retinal layers was conserved even after 35 weeks. No direct correlation was found between donor age or sex in terms of tissue survival. While cones exhibited a gradual loss of outer segments, they did not undergo severe apoptosis, and a mean density of 4000-5000 cones/mm^2 was maintained even in long-term cultures. In the inner retina, subpopulations of bipolar, horizontal, and amacrine cells showed morphology closely resembling the normal state. Additionally, synaptophysin staining unveiled synaptic structures with a similar normal-like morphology. Despite a reduction in the number of ganglion cells, the presence of surviving ganglion cells was confirmed by immunohistochemistry and electrophysiology. The applied viral vectors led to an effective and consistent transduction, that was highly reproducible between cultures from different donors.

Our results show that it is possible to maintain adult human retinas in a suitable culture system for at least 8 months. The long survival time and low inter-sample variability allow pharmacological compounds to be tested on the human retina in a cost- and time-effective manner. In addition, long-term culture allows the delivery of viral vectors and opens up new and efficient strategies for the development and testing of gene therapeutic approaches and can help to reduce the use of animals in both academic and industrial research.

P0383

TRAIL-Mediated Increased Insulin Secretion in Insulin-Producing Cells Generated from Induced Pluripotent Stem Cells

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Generating pancreatic beta cell-like insulin-producing cells (IPCs) from induced pluripotent stem cells (iPSCs) is a promising strategy to compensate for beta cell loss in diabetes. Despite recent advances, developing more efficient and safer protocols remains crucial. We have included TNF-related apoptosis-inducing ligand (TRAIL) in the differentiation protocol of IPCs from human iPSCs and investigated its effects on the generated IPCs.

iPSCs were acquired from human foreskin fibroblast cells by reprogramming via integrating and non-integrating lentiviral vectors encoding Oct4, Sox2, and Klf4 (Lenti-OSK), along with sodium butyrate as a supporting small molecule. The pluripotency of the resultant iPSC colonies was confirmed via cell surface and nuclear marker expressions, and embryoid body analysis. Karyotype analysis and mycoplasma testing were also performed. TRAIL was included in its soluble form in the last stage of the differentiation process, along with the combination of small molecules necessary to induce definitive endoderm (activin A, CHIR99021) and pancreatic differentiation stages (dorsomorphin, retinoic acid, SB431542, forskolin, dexamethasone, ALK5 inhibitor II, nicotinamide, sodium chromoglycate).

Including TRAIL in the differentiation protocol resulted in significantly increased insulin and c-peptide secretion in a dose-dependent manner and an increase in glucose-stimulated insulin secretion (GSIS). We previously correlated TRAIL with a proliferative and protective effect on rodent beta cells. This mechanism may indicate an additional key point in the yet not completely understood multifaceted role of TRAIL as a protective factor in diabetes and underscores its potential further as a therapeutic molecule. We are currently investigating the mechanism of this effect via correlated analysis of predominantly the canonical insulin secretion pathways. Further investigation into TRAIL's mechanisms of action in increased insulin secretion and beta cell proliferation may unveil new therapeutic avenues for diabetes management *(TUBITAK Grant No: 218S617).*

P0384

Viral gene delivery approach to mimic alpha-synuclein pathology in a preclinical model for Parkinson’s disease

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Gene therapy has become a game changer in treating diseases with high unmet medical needs. Beyond its therapeutic potential, viral gene delivery can also serve as a powerful tool for creating translational preclinical models.

Phosphorylation and aggregation of alpha-synuclein (α-syn) in combination with dopaminergic loss are central to the pathology of Parkinson's disease (PD). The A53T point mutation in the SNCA gene, identified in rare forms of familial PD, is known to enhance α-syn oligomerization and aggregation. To investigate these mechanisms, we generated a mouse model using an adeno-associated virus (AAV)-based approach to recapitulate core features of PD, including A53T α-syn-related pathologies. This model will facilitate the study of downstream effects and serve as a valuable resource for developing new therapeutics.

For this purpose, 13-week-old C57BL/6J mice received a unilateral injection of AAV carrying the human A53T mutated SNCA gene encoding α-syn protein (AAV-hA53T) into the substantia nigra (SN). The contralateral SN was injected with an AAV-empty control vector. After 8 weeks, the brain was collected and subjected to biochemical and histological analyses.

Increased α-syn expression driven by the AAV vector in the ipsilateral SN was successfully proven using a Mesoscale Discovery (MSD) immunosorbent assay and quantitative immunofluorescence. In addition, quantitative rater-independent immunofluorescence analyses demonstrated significantly elevated levels of α-syn phosphorylated at serine 129 in the SN. However, murine α-syn levels were not affected in the striatum.

Dopaminergic loss could be proven as well by detecting a significant loss of tyrosine hydroxylase (TH)-positive soma and fibers in the caudate putamen (CPu) and SN. Additionally, dopamine transporter (DAT) signal was significantly lower in the AAV-hA53T-injected ipsilateral SN compared to the contralateral SN. Inflammatory processes were also evaluated showing not only microgliosis upon AAV-hA53T injection but also brain infiltration of CD3-positive T-cells and more specifically cytotoxic CD8-positive T-cells. Furthermore, increased lipid peroxidation was observed in the CPu and SN of AAV-hA53T-injected hemispheres, as demonstrated by immunolabeling for malondialdehyde.

In summary, utilizing viral gene delivery to replicate the core features of PD allows for the detailed study of A53T α-syn-related pathologies and downstream signaling. By replicating key pathologies such as α-syn accumulation, dopaminergic loss, lipid peroxidation, and immune cell infiltration, the AAV-hA53T mouse model serves as a valuable tool for analyzing the molecular mechanisms underlying synucleinopathies. This model holds significant potential for paving the way for the development of novel therapeutic interventions.

P0385

Characterization of Usher syndrome 1B human retinal organoids derived from *MYO7A* knockout hiPSCs

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Usher syndrome (USH) is an autosomal recessive disorder characterized by vision loss due to retinitis pigmentosa, hearing loss, and impaired balance. Usher syndrome type 1B (USH1B) is the most severe USH subtype caused by mutations in the *MYO7A* gene that encodes a motor protein

expressed in various human tissues, including the retina, inner ear, kidney, and lung. In rod and cone photoreceptors, MYO7A is crucial for transporting opsin through the connecting cilium. In the retinal pigment epithelium, MYO7A regulates the movement of melanosomes and phagosomes. Currently, there is no effective therapy for USH1B because of the lack of a faithful animal or cellular model and the limited knowledge of the pathomechanisms and function of the MYO7A protein. The establishment of human USH1B retinal organoid models would therefore contribute to a better understanding of this disease and to the testing of new therapeutic approaches. Here, we investigated retinal organoids derived from *MYO7A* knockout hiPSC. Gene knockout was achieved using the CRISPR-Cas9 technology designed to delete the genomic sequence between exons 9 and 12 in the *MYO7A* gene in human induced pluripotent stem cells (hiPSCs). In the retinal organoids (ROs) generated from *MYO7A* knockout hiPSCs we observed a significant reduction in the number of the retinal pigment epithelial cell (RPE) patches. Moreover, the RPE patches showed a delay in pigmentation. In addition to the RPE phenotype, we detected a significant reduction in the neural epithelial layer´s thickness around differentiation day 120. A more comprehensive investigation of the RPE- and photoreceptor-related phenotypes in the *MYO7A* knockout ROs is still in progress. Our results so far suggest that we have succeeded in developing a cellular model for USH1B that is suitable for analyzing disease mechanisms and testing new therapeutic approaches for this disease.

P0386

Lentiviral delivery of prime editing components to create an in vitro epilepsy model

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Dravet syndrome is a severe form of epilepsy in infancy characterized by frequent seizures and caused by mutations in the SCN1A gene leading to haploinsufficiency. This research focuses on the delivery of prime editing (PE) components into a neuroepithelial-like cell line (AF22) to create a heterozygous loss-of function mutation in the SCN1A gene to generate an efficient in vitro model to test novel therapies on. Initially, plasmid transfections of the prime editor and prime editing guide RNA (pegRNA) achieved up to 20% allele-specific editing for the target mutation in HEK293T cells. We previously evaluated efficacy of PE2max and PE4max editors in HEK293T cells that showed no significantly difference of editing efficiency. Subsequently, we tested if efficacy could be improved using a single lentiviral plasmid with both the pegRNA and the prime editor (LV-Ef1a-PE2-pegRNA (15.3 kb)). The plasmid is designed with Ef1a driven expression to get a strong expression in the human stem cell (AF22). Single plasmid transfection results in maximum edit percentage of 31% in HEK239T cells. Plasmid transfection failed in AF22, as did lentiviral infection, the latter likely due to plasmid size that may result in low packaging of lentivirus. Next, a lentiviral vector with the smaller novel PE6a was designed including an enhanced pegRNA (epegRNA) and blasticidin resistance gene (LV-Ef1a-PE6a-epegRNA-BSD (11.5 kb)). This plasmid was transfected in HEK293T, achieving a 25% edit rate after 2 weeks of blasticidin selection. Concurrently, lentiviral infections with this vector yielded an edit rate of up to 21% in HEK293T. At this moment, lentiviral infections in AF22 cells are tested. In conclusion, our LV-Ef1a PE2-pegRNA vector can efficiently create a mutation in HEK293T cells using single plasmid transfection, but its size prevent lentiviral packaging. The smaller sized PE6a-pegRNA can be delivered using lentivirus and creates a mutation after antibiotic selection, offering a promising approach for efficient gene editing in other cell lines which are hard-to-transfect, like the AF22 neuroepithelial-like cell. Future steps include the creation of a mutant SCN1A cell line and to test

therapeutic potential and phenotypic features of this cell line after treatment on wild type and mutant strain.

P0387

Enhancing iPSC differentiation into skeletal muscle on alginate scaffolds for regenerative medicine applications

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The generation of muscle tissue from induced pluripotent stem cells (iPSCs) holds significant promise for advancements in regenerative medicine. Our research investigated the efficacy of alginate scaffolds, modified with the cell-adhesive peptide RGD (Arg-Gly-Asp), in supporting the differentiation of iPSCs into striated skeletal muscle cells. This study evaluated both the viability of iPSC-derived cells within these bioengineered matrices and the differentiation potential of muscle progenitor C2C12 cells, aiming to pave the way for future applications in muscle regeneration.

Initial scaffold viability was assessed by seeding GFP-modified HEK293T cells in 2% oxidized, low molecular weight (50 kDa) alginate scaffolds, modified with RGD, through lyophilization technique. The GFP-modified HEK293T cells showed a notable increase in cell numbers by day 5, indicating successful proliferation and scaffold interaction. Considering these results, we cultured C2C12 muscle progenitors in the same scaffolds for 14 days, monitoring cell viability and differentiation into striated muscle cells. Viability assessments using live/dead assays showed a high survival rate of C2C12 progenitors, with 90.06% viability on day 1 and over 87% viability on days 7 and 14. Morphological changes, indicative of differentiation, were observed by day 7, with cells transitioning from a round to an elongated shape. By day 14, cells exhibited a circularity of 0.20 compared with the control group, 0.62, and an aspect ratio (AR) of over 8.5 compared with the control group, 1.45. These results suggest that the progenitors not only interacted with the alginate scaffolds but also utilized the biomaterial as a platform for differentiation.

Encouraged by these findings, we proceeded to culture iPSCs on RGD-modified alginate scaffolds. Preliminary results showed a viability rate of approximately 50% on day 1. This early viability is crucial as it sets the foundation for subsequent differentiation into striated skeletal muscle cells. Our data demonstrate the potential of RGD-modified alginate scaffolds to support the viability and differentiation of iPSCs into striated muscle cells, stressing a significant advancement in the development of regenerative medicine therapies for muscle regeneration.

P0388

MSc and drugs enhance neuroprotective effects on photoreceptors

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Mesenchymal stem cells (MSCs) hold great promise in the field of neuroprotection due to their ability to secrete bioactive molecules through paracrine signalling, offering protection to the retina in degeneration initiated by the retinal diseases. However, enhancing their neuroprotective effects to protect degenerative retinal layers completely including photoreceptors remains a challenge. This research addresses this issue.

Human adipose-derived MSCs were cultivated under standard conditions with 5% CO2 at 37°C in a humidified cell culture incubator. The cells were grown in low glucose DMEM medium supplemented with GlutaMAX™, 10% fetal bovine serum (FBS), and 2% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin). The MSCs were co-incubated with neuroretina using a transwell system and treated with different drugs—vasoactive intestinal peptide, nicotinamide, and retinoic acid—and their combinations in 6-well culture plates for 15 days. The impact of these treatments on the neuroretina was assessed through toluidine blue staining and microscopy.

The results showed significant degeneration of photoreceptors and other retinal layers in the presence of only retinal pigment epithelium (RPE) conditioned medium compared to the 0-hour control. Further assessments revealed that neuroretina co-cultured with MSCs alone partially retained their structure. However, neuroretina co-cultured with MSCs and treated with drugs and their combinations retained their photoreceptors and other retinal structures more intact. The differences in the neuroprotective impacts on photoreceptors and other retinal structures under different culture conditions were significant.

The presence of drugs and their combinations with MSCs proved to be more effective in providing neuroprotection to degenerative neuroretina. These findings offer valuable insights for optimizing the use of MSCs in different stages of retinal degenerative damage, highlighting the enhanced protective effects achieved through combined MSC and drug treatments.

P0389

Zebra Fish model for Hutchinson-Gilford Progeria Syndrome. New therapeutic strategies targeting senescence

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Hutchinson-Gilford Progeria Syndrome (HGPS) is a very rare and fatal disease, characterized by premature aging and death of patients before reaching puberty. In HGPS, accumulation of a truncated form of the lamin A precursor, progerin, occurs, causing structural defects in the nuclear lamina, as well as in the differentiation and proliferation of mesenchymal stem cells (MSCs). To this day, we maintain at CICA a mutant zebra fish line for the *zmpste24* gene, which we will use as a HGPS model as well as senescence pathology model. These animals are transparent for a large part of the time of their development, which allows the study of their organs in a visual and minimally invasive way. In addition, they have a great capacity to regenerate parts of their body, females can produce hundreds of embryos every week and these embryos develop very quickly, which allows for very agile research, especially interesting in the study of our pathology. We have carried out functional studies in the zebra fish HGPS model, such as

identification of markers of senescence (*p53*, *MDM2*, *LMNA*, *P18*, *p27 and CDKN2A/B*), oxidative stress and purine metabolism (*CD13*, *ENO* and *PRPS1*) by genetic and proteomic studies. The senescence markers are statistically significant increase in our HGPS model *vs* wild type. This is the first step towards our goal, which is to use this animal model to refine microRNA microinjection protocols into larvae and to perform shotgun proteomic studies to discover the pathways involved in MSC aging, and therefore that symptoms associated with HGPS and aging are reduced providing better quality of life to these patients.

P0390

Differences in engraftment potential of human CD34+ cells in NSG and NBSGW mice depend on the pre-conditioning method

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Gene therapy for hematopoietic stem cells is a promising therapeutic approach for a variety of different human diseases. For that, humanized mouse models are often used to evaluate novel stem cell-based gene therapy approaches. An important prerequisite is to make the hematopoietic niche available for the transplanted HSCs by reducing the number of endogenous cells in the mouse bone marrow (BM), either by sublethal irradiation or by the myeloablative drug busulfan. Here, we established and compared different conditioning methods for two widely used mouse strains, NSG (NOD.SCID.IL2rγ$^{-/-}$) and NBSGW (NOD.Cg-Kit^{W-41J} Tyr^{+} Prkdc^{scid} Il2rg^{tm1Wjl}/ThomJ), by characterizing the engraftment behavior of transplanted human CD34+ cells. To this end, we tested two doses of busulfan (35mg/kg or 25mg/kg) in NSG mice or preconditioned the mice with irradiation (X-ray; 270cGy). NBSGW mice received hCD34+ cells either without any preconditioning or after a low dose of busulfan (12.5mg/kg). All mice received CD34+ cells (mobilized peripheral blood CD34+ cells; 2x10^{6} cells/mouse) after preconditioning and human cell engraftment was assessed by flow cytometry up to 16 weeks after transplantation. For NBGSW mice, we were able to demonstrate that transplanted stem cells can be engrafted without prior conditioning (20% hCD45+ in the peripheral blood and 60% in the bone marrow). The engraftment rate of the transplanted cells can, to some extent, be improved by mild conditioning with busulfan (50% hCD45^{+} in the peripheral blood and 80% in the bone marrow). In comparison to NBSGW mice, NSG mice also show a high engraftment rate when treated with very high doses of busulfan (70% hCD45^{+} in the peripheral blood and 90% in the bone marrow). However, this dose proved to be unsuitable due to high dropout rates (50%). Better survival was observed with lower doses of busulfan (30% hCD45^{+} in the peripheral blood and 70% in the bone marrow), but with a significantly lower engraftment of the transplanted cells. As irradiation showed significant methodological variability and led to other side effects, such as tooth loss associated with reduced food intake, our results emphasize the need for further studies to establish an appropriate conditioning regimen in NSG mice. In conclusion, busulfan conditioning seems to be a better preconditioning method in NSG mice compared to irradiation. Mild pre-conditioning in NBSGW mice improved human cell engraftment compared to non-conditioned NBSGW mice but appeared to increase anemia. Overall, there are several options to improve the engraftment rate of HSCs in mice, but the higher engraftment rate may be accompanied by higher dropout rates.

P0391

Scalable production of Hematopoietic Stem Cells from iPSCs using stirred tank bioreactors: ensuring batch consistency for cell therapy and regenerative medicine applications

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Hematopoietic stem cells (HSCs) are critical for the development of blood and immune cells, offering immense potential for cell therapy and the treatment of various haematological conditions. The manufacturing of HSCs from induced pluripotent stem cells (iPSCs) with consistent quality and at scale is essential for their integration into therapeutic applications. However, achieving this goal is challenging due to the complexity of the differentiation process. Ncardia, with extensive experience in iPSC innovation, has developed robust protocols for HSC differentiation using stirred tank bioreactors, ensuring large batch consistency and scalability.

Ncardia adapted HSC differentiation processes, originally developed in 2D, into 250mL stirred tank bioreactors, with the goal of scaling up to 1L and 3L bioreactors. A Design of Experiments (DoE) approach was employed to systematically evaluate critical process parameters (CPPs) such as dissolved oxygen, agitation speed, and inoculation density. Parallel testing of multiple differentiation protocols was conducted to establish proof of concept. In-process monitoring, combined with comprehensive data science analysis, enabled a robust understanding of critical quality attributes (CQAs). This methodology ensured the optimization and consistency of the differentiation process, facilitating scalable and reproducible HSC production.

The differentiation protocols developed by Ncardia in stirred tank bioreactors successfully yielded high-quality HSCs characterized by their ability to self-renew and differentiate into various blood and immune cell types. The iPSC-derived HSCs exhibited typical morphology and expressed key markers (CD34, CD45 and CD43). These HSCs showed potential for differentiation into multiple immune cell lineages, including natural killer (NK) cells and macrophages, highlighting their applicability in cell therapy and immunotherapy. Moreover, stirred tank bioreactors showed higher cell yield and viability when compared to the available 2D differentiation methods.

Ncardia's innovative use of stirred tank bioreactors for HSC differentiation from iPSCs addresses the critical need for large batch consistency and scalability in cell therapy applications. By combining expertise in stem cell biology with advanced bioreactor technology, Ncardia ensures high-quality, reproducible HSC production with GMP compliant processes. This capability not only accelerates the development of cell therapies but also enhances the potential for generating diverse immune cell types for therapeutic use.

P0392

Development of an electrically functional 3D retina organ model for drug discovery to be supported by iPSC-derived RPE cells

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Neuroretinal degeneration as Age-Related Macular Degeneration (AMD) and Diabetic Retinopathy (DR) are major causes of blindness worldwide and though treatments are available, no regenerative, curative approaches exist. Moreover, diseases rely on costly and frequent intraocular injections. There is thus a high unmet need for innovative regenerative cell therapies to respond to increasing numbers of patients suffering from neuroretinal degeneration. The development of novel therapeutics necessitates, however, drug discovery tools of highest quality and transferability to patients. Cell culture models lack complexity of the multilayered retina and are thus, only limitedly transferable. On the other hand, animal testing lacks transferability due to species differences and raises ethical concerns. Our purpose is the development of an electrically functional 3D retina model co-cultured with iPSC-derived RPE cells from healthy and diseased human donors offering new ways in drug development. Here, we present ERGs of pig retina measured up to 48h of co-culture with pig RPE cells.

Porcine eyes were received from a slaughterhouse 5-8h *post-mortem* and RPE cells isolated and grown until 60% confluence under standard conditions (DMEM/F12, 10% FBS, 1% antibiotics and anti-fungicides). At around 4 weeks, retinae samples were isolated under dim red light from porcine eyes and co-cultured with RPE cells in indirect contact to photoreceptors in culture inserts at 21°C, and Ames' medium supplemented with 5% FBS, 1% N2, and 1% B27.

ERGs were recorded using the Ocuscience ERG ex vivo adapter at 0h, 6h, 24h and 48h of co-culture. The samples were illuminated with flashlights from 30 to 30K mcd*s/m^2 from 4-10 flashes of 2-10s. Normal A and B waves could be recorded in 16 out of 22 retina sample duplicates. At medium intensity (3rd out of 7, 300 mcd*s/m^2), the mean measured A wave amplitudes for the different timepoints were -54±40µV, -29±34µV, -21±14µV, and -2.5±1.5µV, respectively; and 81±56µV, 40±43µV, 35±26µV, and 3±2.3µV, respectively, for the B wave. The results show a decreasing amplitude pattern over time with relevant magnitudes and with statistical significance between first and last timepoint. The mean implicit time for the A wave was 0.36±0.25ms, 0.5±0.3ms, 0.57±0.72ms, 0.9±0.27ms and for the B wave 12.4±9ms, 7.9±4.5ms, 7.6±6.5ms, 7.3±4.5ms. Shown ERG measurements prove activity not only of photoreceptor but also of bipolar cells for 2d offering for the first-time neuronal function testing in the development of personalized regenerative cell therapies, highly transferable compared to in vivo ERG (pig Full-field ERG mean A wave around 40µV). Next, neuronal survival will be preserved by co-culture of the retina with human iPSC-derived RPE cells to enable functional analyses for multiple days. Moreover, the use of iPSC-derived RPE cells from patients will significantly advance midterm drug testing in a highly transferable disease model.

P0393

Validation of an iPSC-derived engineered heart tissue (EHT) model for testing AAV-based therapeutics

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Engineered heart tissues (EHTs) are 3D structures of hiPSC-derived cardiomyocytes embedded in a fibrin hydrogel. They align along force-lines and beat spontaneously, allowing for force and contractile parameter measurement. EHTs are an excellent human testbed to evaluate clinically relevant compounds like viral vectors for their transduction and functional efficacy. We tested three different constructs, AAV2-eGFP, AAV2i8-eGFP, AAV9-eGFP at three different multiplicities of infection (MOIs), $1x10^4$, $3x10^4$, and $1x10^5$. The contractile force and beating frequency of EHTs were measured under sterile, non-invasive conditions using the in-house engineered automated imaging instrument DiNAQUBE™, and eGFP protein levels were evaluated within living EHTs post-transduction using fluorescence microscopy. Further, eGFP mRNA levels were quantified after 14 days post transduction by qPCR. Compared to untransduced control hiPSC EHTs, there were no major differences observed either for contractile force or beating frequency in any of the transduced EHTs with different MOIs. The qPCR analysis unveiled a rise in GFP signal that was time- and MOI-dependent. Further, qPCR data identified AAV2-eGFP as the top-performing vector, followed by AAV9-eGFP, and AAV2i8-eGFP exhibiting the lowest performance. This trend was consistent with GFP levels observed within living EHTs post-transduction using fluorescence microscopy. In summary, EHTs can be effectively transduced by clinically applied AAV serotypes without negatively influencing their physiology and function. Therefore, the EHT system provides a human screening platform for characterization of AAV therapeutics targeting genetic and acquired cardiac diseases.

P0394

Functional validation and therapeutic target identification in neurodevelopmental disease models using hiPSC-derived CNS cells and forebrain organoids

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This study presents the functional validation process of CNS cells and forebrain organoids derived from patient-induced pluripotent stem cells (hiPSCs), and CRISPR-Cas9 edited hiPSC lines. The aim is to better understand neurodevelopmental diseases caused by rare genetic variants and seek for potential therapeutic targets.

Specifically, hiPSCs were differentiated into 3D brain organoids and 2D cortical neurons, which mimic the structure and function of the human brain at various developmental stages.

We conducted protein extraction and analysis from cortical neurons and brain organoids at multiple differentiation points, revealing significant expression differences between healthy controls and patient-derived/edited cells. These differences provide insights into the molecular mechanisms underlying neurodevelopmental disorders and suggest potential therapeutic pathways. These findings pave the way for developing advanced therapies tailored to the unique genetic background of patients with these rare neurodevelopmental disorders.

Ongoing studies focus on neuronal migration and ion movement within neurons, crucial processes for proper brain development and function. By understanding how these processes are disrupted in patient-derived cells, we aim to identify novel intervention points for therapeutic strategies.

Additionally, we have collaborated on delivery methods (LNP and AAV) to target these models, with promising LNP results showing successful CRISPR-Cas9 delivery into stem cells, leading to targeted gene editing. We are currently expanding our studies to include both 2D cortical neuron cultures and 3D brain organoids, evaluating the potential of these systems to deliver therapeutic agents precisely and effectively.

P0395

Novel Therapeutic Approaches for Pediatric Myelodysplastic Syndromes: Exploring Base Editing in SAMD9/SAMD9L-Associated Disorders

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A 3-year-old boy from Guatemala presented to our hospital with severe hepatitis and trilinear cytopenia. Initial microbiological tests ruled out infectious diseases, and clinical and laboratory findings did not meet the diagnostic criteria for Hemophagocytic lymphohistiocytosis. A bone marrow (BM) histological biopsy confirmed the diagnosis of Refractory Cytopenia of Childhood (RCC), a rare form of pediatric Myelodysplastic Syndrome (pMDS) characterized by impaired hematopoiesis, BM hypocellularity, and a high risk of progression to acute myeloid leukemia.

Clinical exome sequencing specific for pMDS revealed a germline mutation in the Sterile α motif domain-containing protein 9 (SAMD9) gene (c.4496A>G), which was classified as a Variant of Uncertain Significance (VUS). SAMD9 and its paralogue SAMD9L, both located on chromosome 7, are the most common genes associated with pMDS predisposition. These proteins are crucial for maintaining hematopoietic homeostasis, particularly under IFN-α regulation. Gain-of-function (GOF) mutations in SAMD9/SAMD9L can enhance anti-proliferative and pro-apoptotic effects in hematopoietic stem cells.

To elucidate the pathogenic role of the SAMD9 mutation (c.4496A>G) found in our patient, we employed a G>A base-editing approach in K562 cell lines, which possess triploidy of chromosome 7. This model allowed us to investigate the cumulative effects of 1, 2, or 3 edited SAMD9 alleles. The mutation was successfully introduced in a bulk population of K562 cells with minimal off-target effects. We then selected and expanded cell clones carrying 1/3 or 2/3 edited alleles.

Notably, cells with 3/3 mutated alleles were unable to proliferate, indicating a potent anti-proliferative effect induced by the mutant SAMD9 protein.

Preliminary experimental results indicated that cells carrying the SAMD9 mutation exhibited increased SAMD9 expression. These cells also demonstrated increased susceptibility to inflammatory stimuli and higher levels of apoptotic cells compared to wild-type cells. Additionally, cellular growth assays revealed a significant decrease in proliferation in mutated cells following IFN-α stimulation. These findings corroborate the hypothesis that SAMD9 mutations lead to excessive anti-proliferative and pro-apoptotic responses under inflammatory conditions, contributing to the pathogenesis of RCC in pMDS.

Our study demonstrates the utility of base-editing technology in modeling and validating the pathogenic impact of SAMD9/SAMD9L variants in vitro. The increased expression of SAMD9 and heightened apoptotic response in mutated cells highlight the potential role of this mutation in disrupting hematopoietic homeostasis.

In conclusion, base-editing represents a powerful tool for investigating the functional consequences of genetic variants in pMDS. This approach not only enhances our understanding of disease mechanisms but also holds promise for developing targeted therapeutic strategies, such as base-editing to reverse pathogenic mutations in pMDS. Our findings provide a foundation for future research into precision gene-editing treatments for genetic hematopoietic disorders.

P0396

Proof-of-concept for a one-step CRISPR-based gene therapy approach for STAT1 gain-of-function

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Inborn errors of immunity (IEIs) are rare inherited disorders affecting the immune system, presenting increased susceptibility to pathogens and are often associated with severe non-infectious comorbidities. One of these IEIs is called autosomal dominant signal transducer and activator of transcription 1 (STAT1) gain-of-function (GOF). Up to now more than 110 mutations leading to STAT1 GOF were identified with very variable and mostly unexplained phenotypes. STAT1 is a pivotal transcription factor in the immune response. Allogeneic hematopoietic stem cell transplantation is the only curative option for now but it is accompanied by high morbidity and mortality. Alternatively, gene editing of autologous hematopoietic stem cells might offer a viable curative treatment for the patients given that STAT1 GOF is a monogenic disorder. Here, we provide proof-of-concept for therapeutic gene knock-in in the *STAT1* locus. Our strategy relies on engineered virus-like particles for delivery of CRISPR/Cas9 components combined with adeno-associated viral vectors to provide a donor template. Exploiting the homology-directed repair mechanism after Cas9-induced double-strand break, we report successful targeted integration in the *STAT1* locus, endogenous regulation by *STAT1* promoter, and partial rescue of STAT1 function. Our study highlights the potential of CRISPR/Cas9-mediated gene editing therapy for patients with any mutation causing STAT1-related disorders.

P0397

Haematopoietic stem cell prime editing for the rescue of B cell development in X-linked agammaglobulinemia

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X-linked agammaglobulinemia (XLA) is a primary immunodeficiency disorder characterised by the absence or severe depletion of mature B lymphocytes, resulting in immunoglobulin deficiency. XLA is caused by mutations in the gene encoding Bruton's tyrosine kinase (BTK), a non-receptor tyrosine kinase critical for B cell maturation and the proliferation of peripheral B cells. BTK loss-of-function therefore leads to a maturational arrest of B cell development, preventing the formation of antibody-secreting plasma B cells. The current standard of care for XLA, which comprises the regular administration of exogenous immunoglobulins, has improved patient outcomes; however, many patients still experience severe breakthrough infections that can lead to an impaired quality of life and fatal complications. The emergence of gene therapy has raised the possibility for restoring *BTK* expression in XLA; despite this, lentiviral vector-based gene addition approaches are not appropriate within this context, given the risk of oncogenic transformation with supraphysiological levels of *BTK* expression. We previously described a Cas9 nuclease-based gene editing strategy in haematopoietic stem cells (HSCs) relying on the insertion of a therapeutic *BTK* cassette encoded by an adeno-associated virus 6 (AAV6) vector in an early exon of the *BTK* locus. Therapeutically relevant editing efficiencies >30% were achieved in patient HSCs, and edited XLA HSCs restored the B cell lineage compartment when transplanted in immunodeficient mice. Despite this, edited HSCs exhibited significantly lower engraftment when compared to a wild-type control. We and others have observed that gene editing protocols based on a nuclease-induced double-strand break with the delivery of an AAV6 donor template trigger a sustained p53-mediated DNA damage response in HSCs, which hinders the translational potential of such approaches. Recently developed double-strand break-free editing approaches such as prime editing can mediate precise nucleotide modifications of all types whilst maintaining the endogenous regulation of the target gene. Here, we report on the application of prime editing for the therapeutic correction of HSCs from an XLA patient harbouring a specific point mutation in *BTK*. Initial candidate selection and optimisation is performed on an 'XLA-like' B cell line developed in-house, followed by editing of patient HSCs. This approach is compared to the Cas9-AAV6 approach in terms of editing efficiency, HSC stemness, genotoxicity, and phenotypic rescue assessed using a B cell differentiation and functional assay platform. Further xenotransplantation experiments in immunodeficient mice will shed light on the repopulation ability of prime-edited HSCs in comparison to the Cas9-AAV6 approach and will pave the way for future preclinical studies to translate a gene editing approach for XLA into the clinical setting.

P0398

Novel automatic application for enrichment of CD34+ cells from apheresis products using the CliniMACS Prodigy

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Hematopoietic stem cell transplantation (HSCT) is a curative treatment for many hematologic malignant and non-malignant diseases both in allogeneic transplantation and in the context of gene therapy. To this end, the number of the transplantable CD34+ cells as well as the purity of the graft are critical parameters for these therapeutic approaches. Lately, the stem cell boost has also emerged as a potent treatment modality not only in the classic HSCT field but in the context of CAR-T cell therapy as well, as a measure to manage cytopenias.

Based on MACS Technology, the CliniMACS Prodigy LP-34-(320) System enables automatic production of an injectable CD34 enriched cell population which is simultaneously passively depleted by allogeneic reactive T cells. Central components of the new application, which utilizes tubing set TS 320, include a newly developed platelet removal step, a WBC-adjusted separation and the possibility of product release at a requested time point because the process allows storage of the labeled cells for up to 16h. The process is sufficient for the enrichment of 1.2e10 CD34+ cells from up to 120e10 total WBC. A new fluorescent flow analysis protocol suitable to analyze very low percentage of remaining T cells in the final product and a low percentage of CD34+ cells in the starting material was additionally developed.

In house evaluation runs (n=22) using mobilized leukapheresis products resulted in a mean depletion of 4.5 log (range 3.8 – 4.9) for T cells, a mean platelet depletion of 2.8 (range 2.2 – 3.7), a mean B cell depletion of 3.2 (range 2.1 – 3.5), and a mean purity of 91.2% (range 81.1 – 96.1) in the CD34 enriched product. Viability of CD34+ cells in the target product was above 99%, mean yield was 65% and mean WBC recovery 82%. The process duration is approximately within 3.2 – 5.5 hours, depending on the cell numbers, excluding the optional in-process storage.

The novel automated CliniMACS Prodigy LP-34-(320) enrichment process is a regulatory compliant, fully automated process in a closed fluidic system, capable to enrich CD34 and to deplete T cells efficiently from apheresis products. The system delivers ready to use stem cell grafts by giving a flexible process end time and minimal hands on, enabling staff resource planning easier and more convenient. In the CD34 enriched fraction the mean CD34 purity was 91%, the mean CD34 yield was 65% and the CD34 viability was above 99%. The comparability to the existing CliniMACS Plus system was shown in comparison runs. The submission to an European notified body for CE certification is an important next step. In addition, the system is planned for market release as GMP in Autumn of 2024.

Conflict of Interest statement: All authors are employees of Miltenyi Biotec and / or Miltenyi Biomedicine.

P0399

In vivo interrogation of chromatin modifying enzymes during early stages of HSPC clonal expansion and differentiation dysregulation using an AAV8 editing platform

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Hematopoietic stem cells (HSCs) are multipotent adult stem cells responsible for maintaining the blood system. HSCs can self-renew and differentiate into more lineage-restricted progenitors and mature cells. As individuals age, HSC self-renewal and differentiation programs frequently become more dysregulated. One outcome of this age-associated dysregulation is the development of Acute Myeloid Leukemia (AML). AML is one of the most aggressive adult leukemias, with a 25% five-year survival rate in individuals over the age of 65. AML is thought to originate and then evolve from a premalignant ancestral clone, known as the preleukemic HSC. Sequencing of patient tumors has revealed that some of the earliest acquired mutations in HSCs are found in chromatin modifying enzymes. These enzymes play an instrumental role in regulating gene expression through manipulation of chromatin accessibility. Although chromatin modifying enzymes are believed to play an important role in HSC dysregulation in AML, and in aging, their individual contributions to early stages of HSC dysfunction have been difficult to assess *in vivo*. Therefore, we aimed to develop and deploy a multiplexed AAV gene editing approach to determine the role of specific chromatin modifying enzymes in aberrant HSC proliferation and/or blocks in their differentiation, two hallmark phenotypes of preleukemia. We hypothesize that the loss of function of chromatin modifying enzymes in HSCs is necessary to induce preleukemic phenotypes. Through this work we have develop an *in vivo* model system supporting modular targeting of loci in HSCs and direct in vivo characterization of the premalignant stages of HSCs.

P0400

Genome integrity of gene-engineered hematopoietic stem and progenitor cells for the treatment of RAG1 deficiency

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Genome editing using CRISPR-Cas9 is a highly promising tool with the potential to revolutionise the treatment of human genetic disorders. Despite improvements in the technology, assessment of safety and genome integrity after editing procedures is extremely crucial before moving to the clinical translation.

In this study, we aimed to develop an effective and safe *ex vivo* therapeutic strategy in human hematopoietic stem and progenitor cells (HSPCs) for the treatment of recombination-activating gene 1 (*RAG1*) defects, causing severe immunological disorders. We established a combined "knock-out and knock-in" CRISPR-Cas9 strategy to disrupt the mutated *RAG1* gene by non-homologous end joining and simultaneously insert a codon optimized corrective cassette by homologous-directed repair (HDR) process exploiting adeno-associated vector serotype 6 (AAV6) or integrase-defective lentiviral vector (IDLV) as delivery platforms. Thus, our strategy allowed *RAG1* gene correction with efficiencies up to ~30% in HSPCs derived from healthy donors (HD) or *RAG1*-deficient patient, preserving *RAG1* physiological regulation. Indeed, corrected-HSPCs validation through three-dimensional thymic artificial platform and *in vivo* transplantation resulted in the overcome of T and B cell differentiation block, which is observed in patients carrying *RAG1* mutations.

To evaluate the safety of our approaches, we nominated off-target sites based on *in silico* prediction and on the cell-based assay GUIDE-Seq. High-throughtput sequencing evaluation of putative off-target sites was performed in HD-derived HSPCs (n=3). The sequencing analysis resulted in no indels identification above the noise threshold in both predicted and nominated GUIDE-Seq off-target regions, suggesting a safety profile of our selected nuclease.

To further investigate the genome integrity at the targeted cutting site, we optimised a copy number variation (CNV) assay using droplet digital PCR on *RAG1* edited HSPC-derived colonies. CNV analysis on two adjacent *RAG1* genes, *TRAF6* and *RAG2*, respectively located 56kb upstream and 13kb downstream of the cutting site, showed the occurrence of potential unintended deletions nearby the on-target site, with a higher number of monoallelic deletions in the *TRAF6* gene (~8%) compared to the *RAG2* locus (~2%). For a more unbiased assessment of genomic rearrangements, we evaluated the presence of large structural variants (SVs) at low variant allele fraction (VAF) exploiting the genome-wide, high-resolution optical genome mapping (OGM) approach in edited HD-derived HSPCs (n=2). A dual variant annotation pipeline was used for identifying differences between edited and untreated samples. The resulting data showed the presence of insertions (10-20 kb) and/or inverted duplication, with a VAF of 0.05-0.15 and 0.16 respectively with high confidence, upon editing at on-target site. Additionally, CAST-Seq was performed to discriminate relevant events detected by OGM and CNV-based assay. Importantly, we confirmed the absence of detectable off-target events. However, the presence of large deletions/inversions within the 10kb region surrounding the *RAG1* cutting site were observed. Chromosomal rearrangement assessment in long-term edited HSPCs derived from transplanted immunodeficient mice is ongoing.

Overall, these analyses will be instrumental to shed light on the mechanisms and the impact of the editing procedure on HSPCs and further implement the *RAG1* HDR-mediated strategy before moving to the clinical setting.

P0401

Investigating the biological properties of circulating Hematopoietic Stem/Progenitor Cells in pediatric subjects as novel source for Gene Therapy

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Hematopoietic Stem Progenitor Cells (HSPC) are an heterogenous population of multipotent stem cells which sustains the daily hematopoiesis. HSPC mostly reside within the bone marrow (BM), the principal postnatal hematopoietic organ. Nevertheless, few circulating HSPC (cHSPC) are found in peripheral blood (PB), in physiological states, and their number is increased in pediatric subjects. Our group recently showed that human adult-derived cHSPC present multi-lineage differentiation potential and can home into the BM of xenotransplanted immunocompromised mice, contributing to the short-term hematopoietic reconstitution. However, as conventional HSPC collection procedures remain challenging in very young patients (0-1 years old), the biological features and the clinical value of pediatric cHSPC as cell source for HSPC-based Gene Therapy (GT) are yet to be estimated. Immunophenotyping a cohort of 72 healthy donors (HD) from 0 to 87 years old, we observed increased levels of cHSPC (3.7-fold) in the pediatric group during the first year of life. Moreover, very young HD displayed comparable frequencies of phenotypic Hematopoietic Stem Cells (HSC) and Multipotent Progenitors (MPP) subpopulations to Cord blood (CB), showing also increased clonogenic potential vs. adult HD. In an optimized *in vitro* multi-differentiation assay, single pediatric PB-HSC/MPP preferentially produced lymphoid and erythroid cells, while adult-derived PB-HSC/MPP are skewed towards the erythroid output. In addition, pediatric primitive cHSPC display higher differentiation efficiency than cells isolated from adults. To investigate the molecular features of pediatric cHSPC, we transcriptionally profiled 38,888 pediatric $CD34^{+}$ LIN^{-} cells obtained by different HSPC sources, including BM, PB, mobilized PB and CB. Single-cell RNA-Seq analyses indicate that pediatric cHSPC contain fewer actively dividing cells than the other HSPC sources. Notably, we observed a comparable fraction of transcriptionally-defined primitive cells across all the pediatric HSPC sources. The most primitive HSC from all the HSPC sources showed a similar expression of genes involved in the regulation of cell stemness, self-renewal and quiescence. Furthermore, preliminary data indicate that pediatric cHSPC can be efficiently transduced with lentiviral vectors and expanded *ex vivo*, similarly to CB-HSPC. Transplantations of transduced cHSPC in immunocompromised mice are ongoing, and they will allow estimating their capability of homing into the BM and contributing to the long-term reconstitution of a functional hematopoietic system. Overall, this project will explore the biological properties of pediatric cHSPC, estimating their potential as a novel stem cell source, for both *ex vivo* and *in vivo* HSPC-based GT approaches.

P0402

Intracellular iron overload and defective stromal niche impair hematopoietic stem cells in sickle cell disease

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Sickle cell disease (SCD) is a monogenic disorder caused by a single-nucleotide substitution in the β-globin gene, resulting in chronic hemolytic anemia, iron overload (IO), vaso-occlusion episodes associated with severe pain and inflammation and multiple organ damage. Transplantation of autologous hematopoietic stem cells (HSCs) genetically-modified by gene addition or editing represents a promising curative strategy. The bone marrow (BM) niche influences the quality of the harvested HSCs for *ex vivo* manipulation and their subsequent engraftment and reconstitution capacity once transplanted in patients, thus impacting the degree of clinical benefit. Recent works showed an impaired function of HSCs in SCD murine models, but a deep characterization of the molecular and cellular players involved remains unexplored. We hypothesized that IO, oxidative stress and inflammatory cytokines might impair directly HSCs or damage BM niche cell populations, thus interfering with the maintenance of HSCs and potentially compromising the clinical outcome.

We found intracellular and mitochondrial IO in HSCs from SCD Townes mice (*SS*), correlating with high ROS levels. As a result, mitochondria in HSCs are impaired with low activity and ATP production. *In vivo* administration of the iron chelator DFO normalized intracellular iron content and mitochondrial activity in HSCs, demonstrating that IO causes mitochondrial defects and metabolic adaptation. Ongoing HSC transplantation experiments will reveal whether reduction of intracellular IO could restore the impaired *SS* HSC frequency and function.

Multiparametric flow cytometry analysis of BM stromal components revealed reduced frequency of mesenchymal stromal cells (MSCs) in *SS* mice. Moreover, MSCs from SCD patients showed increased oxidative stress and reduced proliferation *in vitro*, with a potential impact in their support to HSCs. Indeed, SCD MSCs were less efficient than HD in maintaining primitive HD $CD34^{+}CD45RA^{-}CD90^{+}$ HSPCs, leading to their differentiation after *in vitro* co-culture experiments. Further analyses will unravel the ability of impaired SCD MSCs to sustain the engraftment and reconstitution of edited SCD HSPCs.

Overall, we unravelled an unexpected role of iron on mitochondrial metabolism and function of SCD HSCs. Iron and other stressors can affect HSCs both directly and indirectly by acting on MSCs, which in turn fail to preserve HSCs. A deeper investigation of defective HSC-niche cross-talk is currently ongoing to optimize the outcome of HSC transplantation and gene editing in SCD.

P0403

Disease features of hematopoietic stem cell affect correction of beta-thalassemia in gene therapy treated patients

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Stress conditions affect hematopoietic stem cell (HSC) fate and blood lineage output, indicating that haematopoiesis is flexible to adapt to different stimuli. In beta-thalassemia (Bthal), the severe anaemia and the ineffective erythropoiesis generate a chronic stress status of the bone marrow (BM) microenvironment which resulted altered both in cellular and molecular components, affecting HSC biology. In Tiget-Bthal clinical trial, HSC lentiviral mediated gene therapy (GT) resulted in transfusion independence in patients with a positive correlation between proportion of *in vivo* engrafting corrected HSCs and degree of anaemia correction (Marktel et al. 2019). Variability in terms of HSC transduction and *in vivo* reconstitution poses limitations for clinical outcome and the status of BM microenvironment might influence the quality of HSC, harvested for genetic engineering, and their engraftment and reconstitution capacity once transplanted.

Our transcriptome profile, from bulk RNAseq and scRNAseq, highlighted that Bthal BM HSCs/MPPs (hematopoietic stem cells/multipotent progenitors), differently from healthy donor ones, are in an activated state and primed towards erythroid differentiation trajectory. Consistently, HSCs/MPPs showed altered pathways related to stemness along with a low expression of a dormancy transcriptional signature. We hypothesize that specific biological features of Bthal HSCs can influence their repopulating capacity and the consequent hematopoietic reconstitution in the GT treated patients. Therefore, we focused on two GT patients (Pts) with opposite clinical outcome. Pt5, despite having high VCN (vector copy number/cell) in the drug product and adequate conditioning, experienced an unexpected VCN drop in all lineages and a consequent lack of clinical benefit, requiring restoration of transfusion support. In contrast, Pt4 showed high engraftment of gene-modified cells resulting in a favourable clinical benefit.

We profiled single hematopoietic stem and progenitor cells (HSPCs) and HSCs/MPPs isolated from BM $CD34^+$harvested before GT (BM pre-GT), from the stem cell source for GT (mobilized peripheral blood $CD34^+$, mPB) and from the BM $CD34^+$ 1yr after treatment.

We identified differences in the composition of hematopoietic subpopulations and in the quiescence signature of primitive compartment before and after GT between the patients, as well as in mPB. scRNAseq analysis revealed a low dormant score in mPB HSCs/MPPs of Pt5 compared with Pt4 before GT. In contrast at 1yr after GT, Pt5 BM HSCs/MPPs were more quiescent, with an enrichment of signatures corresponding to dormancy, hypoxia and TNFα via NFkB signalling. In line with this, integration site analysis retrieved low number of active HSPCs in Pt5. Overall, these data suggest that the degree of HSC dormancy in the stem cell source is important to

preserve their function and hematopoietic reconstitution capacity. Ongoing analysis will highlight whether and how stress signals regulate the dormancy/activation of HSCs/MPPs and their ability to reconstitute the hematopoietic process in GT Bthal patients. The identification of specific signatures of Bthal HSC/MPPs will offers new insight for the prediction of benefit and optimization of protocols in clinical GT trials.

P0404

Optimized transduction of mPB CD34+ cells increases transduction efficiency and VCN while preserving stemness: towards a Phase IIb clinical trial of gene therapy for TDT beta-thalassemia

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Transfusion-dependent ß-thalassemia is a disorder due to mutations in the gene encoding the ß-globin chain causing a reduced or absent production of hemoglobin A, leading to severe anemia and lifelong transfusion dependence. *Ex vivo* hematopoietic stem cell (HSC) gene therapy is an effective alternative cure to allogeneic bone marrow (BM) transplantation. In 2015, we started a clinical trial based on autologous mobilized HSC transduced by the GLOBE vector and administered following a reduced myeloablative conditioning in adult and pediatric patients, and we reported encouraging initial results (Marktel et al. NatMed, 2019)

All patients are alive and well with no adverse events related to gene therapy at the latest follow-up time point. Results from the long term follow up study show a persistent and polyclonal engraftment of genetically engineered cells in most of the patients. The clinical outcome in adult patients confirmed a significant reduction in transfusion requirement while the pediatric population includes both transfusion independent and dependent patients. Overall, the results highlighted the need for a robust *in vivo* vector copy number/cell (VCN) and marking efficiency in HSC to achieve the complete correction of anemia and associated features.

Thus, we improved the manufacturing by using transduction enhancers while reducing the culture time, thus preserving the stemness features of transduced engrafting cells.

Among different tested enhancers, the combination of CSH and PGE2, in refined culture conditions, resulted in higher VCN and transduction efficiency (TE) reaching up to 3- and 1.5-fold increase respectively compared to the previously used standard protocol (STD), that were maintained long-term following transplantation in immunodeficient mice, indicating targeting of repopulating HSC. Importantly, evaluation by RNAseq analysis showed absence of significant transcriptional differences between CSH plus PGE2 treated *vs* untreated $CD34^+$ cells.

Interestingly, the CSH plus PGE2 treated most primitive cells showed an upregulation of pathways involved in the maintenance of healthy stem cell pool and an enrichment of transcriptional signature of quiescence.

The optimized protocol has been scaled-up and cell manufacturing tested by our process development laboratory, outperforming STD protocol both in VCN (2.68 *vs* 0.62) and TE (78.7 *vs* 50.0%) evaluation. Similar results were obtained in all performed large scale runs with a mean VCN and TE of 2.45±0.31 and 75.9±8.2 respectively (n=4). A GLP toxicology and biodistribution study in NSG immunodeficient mice is currently ongoing. The manufacturing of the drug products will be performed by the GMP facility of Ospedale Pediatrico Bambino Gesù (OPBG), following a tech transfer of the optimized protocol, which is currently ongoing. The clinical trial application submission, sponsored by Fondazione Telethon, with Ospedale San Raffaele and OPBG as treatment centers, is planned by 1Q25.

Overall, cumulative results led to the definition of new transduction protocol based on the use of CSH and PGE in refined culture conditions enabling the clinical development of a new academic Phase IIb trial.

P0405

In vivo gene editing of hematopoietic stem cells for the treatment of the beta-hemoglobinopathies

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Gene editing therapies provide the opportunity to provide potentially curative therapies for previously untreatable disorders. Significant progress has been made with two recent FDA approvals for sickle cell disease and transfusion-dependent beta thalassemia (SCD and TDT) using *ex vivo* approaches. While these therapies provide significant clinical benefit and are potentially curative, they still rely on genotoxic myeloablative conditioning and complicated cell manufacturing processes. *In vivo* delivery of gene editing therapies offers an opportunity to make these life-changing treatments accessible to many more patients. Orna Therapeutics is pioneering a new approach to genome engineering through the development of gene editing and *in vivo* delivery platforms.

Using computational and wet lab approaches, we have identified and engineered a suite of class 2 type V endonucleases. Type V nucleases can be leveraged to treat hemoglobinopathies, such as sickle cell disease (SCD), through gene induction and delivery to hematopoietic stem cells (HSCs). We have refined the efficiency of our lead editor in primary CD34+ hematopoietic stem and progenitor cells (HSPCs) from healthy donors. Using both structure-based and unbiased engineering approaches, we identified key residues on the guide RNA to improve overall editing outcomes. In parallel, we engineered the type V protein in multiple ways to significantly improve our editing, resulting in combined improvement of editing rates from single digits to >75% in primary HSPCs.

Our type V editors have been designed to be delivered as an all-RNA composition, which enables *in vivo* delivery by lipid nanoparticles (LNP). Utilizing a high-throughput barcoding screening approach with functional mRNA delivery assessment in non-human primates (NHPs), we have identified a suite of LNPs that have tropism to hematopoietic stems cells without the need for a targeting ligand or antibody fragment. In particular, one identified HSC-tropic LNP (LNP-15) showed >70% reporter-positive bulk CD34+ cells and >95% reporter-positive phenotypic LT-HSCs (Lin-CD34+CD90+CD45RA-) in humanized mice. A single dose of LNP-15 in NHPs demonstrated an average 24% reporter-positive HSPCs. Importantly, a single dose of LNP-15 encapsulating a tool Cas9 mRNA and a BCL11a erythroid enhancer targeted-gRNA showed ~20% editing of bone marrow CD34+ cells in humanized mice. By combining our HSC-tropic RNA delivery with our gene editing capabilities, we aim to make a significant impact on the lives of patients suffering from sickle cell disease and beta thalassemia.

P0406

Development of a safe and efficient gene editing platform for the treatment of Wiskott-Aldrich Syndrome

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Wiskott-Aldrich syndrome (WAS) is a rare, X-linked immunodeficiency caused by mutations in the WAS gene, leading to inflammatory complications, increased autoimmunity, and risks of lymphoproliferative disorders and malignancies. While lentiviral gene therapy is effective, full immune and platelet reconstitution is not always achieved. We have recently carried out a proof-of-concept study using a CRISPR-Cas9 and AAV6-based gene editing platform to knock-in a correct human *WAS* cDNA in its own locus in WAS patient-derived HSPCs, providing specificity, toxicity and efficacy data supportive of continued development of the platform to treat WAS.

To further confirm the functionality of our platform in correcting the disease, we have generated a humanized mouse model of WAS (hWAS-KO) where the murine *was* gene is replaced by its human version carrying a nonsense mutation found in patients with severe disease, resulting in loss of WAS protein (WASp). Phenotypic characterization of hWAS-KO mice highlighted the typical functional defects in hematopoietic cells observed in WAS patients, demonstrating the suitability of our newly designed humanized strain as an animal model of the disease. HSPCs collected from hWAS-KO mice could be effectively corrected by our WAS GE platform targeting human *WAS*, which led to restoration of physiological levels of WASp in vitro and in vivo in a competitive transplantation setting. In parallel, we have progressed this gene editing-based therapy to preclinical studies to assess its feasibility in a potential clinical setting. By manufacturing HSPCs at clinical scale with GMP-compliant protocols we achieved reproducible high rates of targeted integration, while preserving HSPCs viability and colony forming efficiency. Cells were capable of long-term engraftment in NSG mice with reconstitution of haematopoiesis and retention of the correct *WAS* gene in all hematopoietic organs. Histopathological analysis revealed no evidence of tumorigenicity or abnormal haematopoiesis in treated mice. A thorough assessment of potential off target edits, chromosomal aberrations and AAV donor vector

integration imputable to the gene editing procedure was carried out with complementing technologies and by tracking the frequency of these events before and after transplantation. Comprehensive integration of the data retrieved at different time points and from in vitro and in vivo samples highlighted the absence of potential genotoxic events and confirmed the safety of the platform. Altogether, our preclinical safety and efficacy data in the humanized WAS murine model and in human patient derived HSPCs provide strong evidence of the feasibility of the platform and support its translation into early phase clinical studies.

P0407

Efficient, durable and specific *in vivo* genetic engineering of human hematopoietic stem progenitor cells

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Achieving in vivo genetic engineering of hematopoietic stem/progenitor cells (HSPC) has the potential to transform treatment of several human diseases with unmet clinical need. A key challenge is reaching a high enough fraction of HSPC to achieve a therapeutic effect while avoiding main off-targets such the liver. We here designed and tested lentiviral vectors (LVs) and virus-like particles (VLPs) equipped with natural or engineered targeting moieties with tropism for human HSPC and obtained therapeutic and durable levels of *in vivo* delivery of gene-editing cargos to human HSPC, in a comprehensive catalogue of preclinical *in vitro* and *in vivo* scenarios, without relying on selective conditioning or redosing and while avoiding human hepatocytes *in vivo*. Most human-tropic envelopes cannot be tested in wild type animals and long-term humanized mice carry a highly skewed human hematopoiesis. Addressing the current lack of reliable experimental models, we will firstly present a high-resolution study of *in vitro* access to resting HSPC subtypes and an in-depth characterization of human HSPC dynamics in NBSGW mice, resulting in the identification of D7-9 post-humanization, as the optimal timepoint for measuring access to human HSPC via intravenous (IV) particle infusion, when we established that engrafted CD34+ cells maintain a physiological composition and remained confined in the bone marrow (BM). We will then summarize the results of 12 *in vivo* studies testing different LV or VLP configurations in multiple scenarios, leading to the generation of a novel potent VLP carrying a CRISPR/Cas9-MLVgag fusion cargo embedded in a Baboon envelope (BaEVTR) hosting a newly designed tail modification and an additional custom-engineered HSPC-tropic decoration (CoD). This BaEVTR-CoD VLP displayed high potency in resting HSPC *in vitro* (~100% editing) as well as therapeutically relevant (>70% editing 1wk after dosing) and durable (>30% stable editing at 16wks follow-up) levels of gene-editing of BM-resident human HSPC upon systemic delivery *in vivo*. We will then present comprehensive data showing that particles equipped with a Nipah virus (NiV) fusogen engineered with binders for CD133 and CoD modifications, combine potency *on par* with BaEVTR with a high specificity for CD133+ cells, as measured *in vitro* upon testing a panel of 8 cell lines and 11 primary human cells as well as *in vivo*, including in scenarios where CD133-positive HSPC were co-infused with CD133-negative cells. Further, we will show that a NiV-CD117-targeted particle can access, *in vivo*, a BM cell population composing as low as 0.3%

of total huCD45+ cells with a 178-fold increased specificity as compared to broadly tropic envelopes. Importantly, we will also present results in the FRG humanized mouse model, demonstrating that our engineered envelopes avoid human hepatocytes upon IV delivery while maintaining on-target potency in human HSPC. Lastly, we will show that our VLPs can be loaded with base editors and therapeutic gRNAs, achieving efficient *in vivo* editing at the BCL11A and HBG1/2 loci resulting in fetal hemoglobin induction in clonogenic progenitors. These results set the basis for developing a first-in-class particle with high efficiency and specificity for *in vivo* delivery of therapeutic payloads to human HSPC.

P0408

Neurological manifestations in Adenosine Deaminase Deficiency: a single center retrospective analysis

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Neurological and behavioural abnormalities have been previously reported in patients with adenosine deaminase-deficient SCID, but in depth studies in the increasing population of patients surviving long-term, treated by hematopoietic stem cell transplantation (HSCT), autologous gene therapy (GT) or enzyme replacement therapy (ERT), are needed.

We gathered retrospective data on 51 ADA-SCID patients followed-up long-term at our centre, treated either by hematopoietic stem cell gene therapy (HSC-GT) (n= 45, median F-U of 5.33 years, IQR 3.09;8.13), allogeneic hematopoietic stem cell transplant (HSCT) (n=2, F-U 3.04, 12.75 years) or enzyme replacement therapy (ERT) (n=4, 17.91, 25.39, 28.66, 32.77 years) who also received up to year 2002 genetically corrected lymphocytes infusions.

We recorded neurological and neuropsychological evaluations, EEG, brain MRI, and hearing tests at baseline and at regular follow-ups as per standard of care.

In the GT cohort, which included the largest sample of the analyzed population, event-free survival curves of time to cognitive impairment, language impairment, behavioral alterations and hearing loss were estimated through Kaplan-Meier curves and the effect of covariates (sex, parents' consanguinity, age at diagnosis, age at GT, ERT duration prior to GT; brain MRI alterations and feeding disturbance prior to GT) was analysed through univariate Cox proportional-hazard regression with Holm's correction. Data on the HSCT and ERT population were presented as descriptive statistics.

Our data revealed that n=34 patients (66.7%) experienced neurological or psychological impairments after treatment.

Among HSC-GT patients, subjects displaying MRI alterations at baseline showed a tendency to be at higher risk of developing cognitive impairment HR 4.64 (95% CI, [0.96-22.34]; p-value=0.0559, adjusted p-value = 0.3916). An early decline in event-free survival rates after 4 and 6 years from GT was observed in cognitive delay (4 yrs=71.25%, 6 yrs=66.5%), language deficits (4 yrs=56.57%, 6 yrs=51.42%) and behavioral alterations (4 yrs=60.72%, 6 yrs=45.33%), while hearing loss appeared later (4 yrs=96.43%, 6 yrs=67.97%). Among the analyzed covariates, only feeding disturbance at baseline was associated with behavioral alterations development (adjusted p-value=0.0095).

In the cohort of patients treated with multiple infusions of gene corrected lymphocytes + ERT, with the longest F-U, both language impairment (manifested by n=4) and cognitive impairment (manifested by n=3), already present at baseline, showed to be worsening at subsequent F-U; hearing loss and behavioral alterations were manifested by n=2. Conversely, in patients underwent HSCT, n=1 developed behavioral alterations and language impairment.

It was documented a high prevalence of neurological and psychological manifestations in ADA-SCID patients already at baseline, not determined by treatment as HSC-GT, HSC and ERT. The progression of neuropsychological manifestations after any treatment indicates that disease-related CNS damage is still proceeding despite immune-hematological correction. It will be important to conduct larger multicentric studies and establish a comprehensive neurological follow-up in view of early rehabilitation.

P0409

Inducible, erythroid specific expansion of transduced hematopoietic stem and progenitor cells using a novel lentiviral gene therapy vector for beta-hemoglobinopathies

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Gene therapy has recently been established as an effective curative approach for beta-hemoglobinopathies. Despite the promising results observed by current gene therapy vectors, there are several important limitations minimizing the overall outcomes of the approach. Among these, the relatively inefficient transduction of long-term hematopoietic stem cells (HSCs) coupled with the need for high vector copy numbers (VCN) to achieve a therapeutic effect, which increases the possibility of insertional mutagenesis, have yet to be resolved.

Scope of this study is to design and assess a new lentiviral vector for the gene therapy of beta-hemoglobinopathies addressing these limitations. This novel vector comprises of a short-hairpin RNA (shRNA) targeting the HBG-suppressor BCL11A, and the FKBP^{F36V}-MPL selection cassette, encoding for a fusion protein able to ignite a growth signal in response to a chemical inducer of

dimerization (AP20187). Transgene expression is driven by a compact erythroid specific enhancer while a recently identified chromatin insulator (C1) is also incorporated. The inducible shRNA-BCL11A-FKBP^{F36V}-MPL (i-shBCL11A) vector was constructed with the addition of a GFP reporter gene for normalization and tracking purposes.

HUDEP-2 cells as well as hematopoietic stem and progenitor cells (CD34+) derived from healthy donors or sickle cell disease and beta-thalassemia patients were transduced at low MOIs (<10) and subsequently cultured in the presence or absence of AP20187. Transduction efficiency, selective proliferation during erythroid and myeloid (granulocytic-monocytic) ex vivo differentiation, as well as γ-globin expression in erythroid cells were evaluated by flow cytometry and HPLC respectively.

In HUDEP-2 cells, the i-shBCL11A vector induced a significant increase in HbF expression (35% vs 8%, vs untransduced). The addition of AP20187 resulted in a 2.8 fold increase of transduced GFP+-cells compared to the untreated group (68%±7,2 vs 24%±7, p= 0,0005) while no effect was observed in the proliferation of cells transduced with the parent shBCL11A vector (minus-FKBP^{F36V}-MPL). In CD34+ cells, transduction rates ranged from 1-30%, depending on the MOI used. The addition of AP20187 to the erythroid culture of CD34+ cells led to an increase in the percentage of GFP^{+}-cells, starting from a 3-fold and reaching an 8-fold increase at the lowest MOI. In parallel, the addition of AP20187 to the myeloid culture of CD34+ cells led up to a 53-fold increase in the percentage of GFP^{+}-cells, the total population of which had an erythroid signature (CD36+/CD235a+) and was negative for myeloid surface markers (CD33-/CD13-). In addition, the γ-globin/β-like-globin ratio at the end of the erythroid differentiation, was increased in the presence of AP20187 (normal CD34+: 0,30 +AP20187 vs 0,18 -AP20187, SCD CD34+: 0,6 +AP20187 vs 0,41 -AP20187, thalassemic CD34+: 3,49 +AP20187 vs 2,25 -AP20187), as evaluated by HPLC.

We have so far shown that the i-shBCL11A vector can achieve reactivation of fetal hemoglobin and selectively expand the corrected cell population in an erythroid-specific manner. Since it has been previously demonstrated that AP20187-mediated activation of FKBP^{F36V}-MPL can be applied both in vivo and in vitro without adverse side effects, we theorize that this approach might overcome the inefficiency of ex vivo gene therapy associated with low transducibility.

P0410

Gene therapy for Artemis-SCID/leaky SCID patients: preliminary data on the first patient included in the French ARTEGENE phase I/II clinical trial

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Genetic deficiency of the endonuclease DCLRE1C/Artemis, a key factor for the Non-Homologous End-Joining (NHEJ) mechanism, causes Severe Combined Immunodeficiency (SCID) characterized by a complete lack of T and B-cells associated with hypersensitivity to ionizing radiations. Allogeneic Hematopoietic Stem and progenitor Cells (HSPCs) transplantation is the gold standard for treating Artemis-SCID. However, graft-versus-host disease and graft rejection, which can lead to post-transplant complications and mortality, are increased in case of HLA incompatibility between the donor and recipient. In this context, gene addition for autologous HSPCs was presented as an alternative therapeutic strategy, and a clinical trial of gene therapy is already ongoing in the United States. Using a different Lentiviral Vector (LV) containing a short intron-less EF1α promoter to express the human DCLRE1C cDNA, we recently opened a European-supported phase I/II clinical trial (NCT05071222) at the Necker hospital in Paris. The first treated patient (P1) had an Artemis-SCID phenotype diagnosed in the context of severe and heavily treated maternal GvHD (steroids, ciclosporin, Ruxolitinib, Thymoglobulin). P1 had a pretransplant history of infection due to immune deficiency, including treatment for pneumocystis, post-rotavirus vaccination gastroenteritis, and undocumented hepatitis. We processed and transduced autologous CD34+ HSPCs from bone marrow and mobilized peripheral blood of P1 to increase the quantity of gene-corrected HSPCs to reinfuse. We deeply analyzed the HSPCs' phenotype and tested the corrected HSPCs' functionality *in vitro* and in an *in vivo* xenotransplant model to evaluate T-cell reconstitution. All experimental results were compared with the engraftment after the transplant in P1 by VCN quantification, T and B-cell reconstitution, and repertoire analysis. We found a good correlation between the clinical study and HSPC functional test results. Transduced HSPCs initiated T-cell rearrangements *in vitro* and restored T-cell differentiation in P1 patients. The VCN number remained relatively consistent during all the *in vitro* cultures and in the patient's PBMC at the early time point post-gene therapy. Our data demonstrate the successful integration of Artemis transgene with the LV strategy, restoring a functional lymphoid compartment in the treated patient, who is now doing well. These promising results support our decision to involve more patients with Artemis deficiency in our ongoing trial, and interim clinical outcomes will be presented in detail at the upcoming congress.

P0411

Development of adenoviral vectors targeted to human hematopoietic stem cells, expressing mutated CXCR4 variants, for efficient and specific transduction of HSC-enriched populations *in vivo*

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Safe and efficient gene correction in human hematopoietic stem cells (HSCs) *in vivo* may provide curative therapeutic options to millions of patients with inherited diseases of the blood and

immune system. However, a significant challenge with current gene editing approaches in HSCs is the need to mobilize them from the bone marrow into the peripheral blood. Following *in vivo* or *ex vivo* editing, corrected HSCs must re-home to their bone marrow niche in sufficient numbers for polyclonal reconstitution of all blood cell lineages throughout the patient's life. Previous studies have highlighted the inefficiency of homing of hematopoietic stem and progenitor cells (HSPCs) to the bone marrow after mobilization. Nonetheless, the efficacy of HSPC homing can be improved through transient ectopic expression of CXCR4, which plays a critical role in guiding HSPCs to and retaining within their bone marrow niches. Recently, we developed an AVID-388 adenovirus vector-based platform that preferentially transduces primitive human HSPC populations *in vitro* and *in vivo* after intravenous vector administration. In this study, we adapted the AVID-388 platform to investigate how primitive human HSPC populations respond to ectopic expression of two CXCR4 splice variants, CXCR4-V1 and CXCR4-V2, as well as various mutated CXCR4 proteins harboring amino acid substitutions. Analysis of primary mobilized human $CD34^+$ cells infected with CXCR4-expressing and control vectors showed that up to 75% of long-term HSCs (LT-HSC) expressed a GFP reporter 24h post-virus infection at a multiplicity of infection (MOI) of 2000 virus particles per cell. *In vitro* transwell migration experiments with primary human $CD34^+$ cells demonstrated that AVID-adenovirus-mediated ectopic expression of CXCR4-V1 but not CXCR4-V2 under the control of the EF1a promoter increases the migration of transduced LT-HSCs toward an SDF-1 gradient. To achieve more specific expression of CXCR4 in the primitive HSC population, we engineered the AVID-CXCR4-V1 adenoviral vectors that express CXCR4 under the control of CD34 gene promoter or a Synthetic HSC-specific promoter (synHSC). Transwell migration experiments showed that ectopic expression of CXCR4-V1-D262N/A175F mutant under the control of synHSC-specific promoter, but not CD34 gene promoter, improves migration of transduced human $CD34^+CD38^-CD45RA^-CD90^+$ LT-HSC cells toward an SDF-1 gradient *in vitro*. The efficacy and selectivity of gene transfer to human HSPCs and LT-HSCs by the AVID-CXCR4 adenoviral vector *in vivo* will be tested in a humanized mouse model grafted with human $CD34^+$ cells and a non-human primate model. Because AVID vectors can accommodate all currently available gene editing technologies, the HSC-targeted CXCR4-expressing AVID platform has the potential to enable safe and effective therapeutic gene editing of human hematopoietic stem cells *in vivo*.

Conflict of interest statement: DSH is a co-founder, shareholder, and officer of AdCure Bio, LLC, which develops adenovirus technologies for therapeutic use. This work was supported by funds from the US NIH grants AI107960 and CA267694, as well as contracts from AdCure Bio, LLC.

P0412

Biological properties and clonality of engineered hematopoietic stem/progenitor cells persisting long-term after gene therapy

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Human hematopoietic stem/progenitor Cells (HSPC) are successfully used in *ex vivo* hematopoietic stem cell-based gene therapy (HSC-GT) for the treatment of genetic or acquired disorders. Nowadays, >400 patients have been treated in HSC-GT clinical trials showing sustained benefit and safe profile, especially when lentiviral vectors (LV) are used as the delivery platform. These numbers will increase in time with HSC-GT therapies now available in the market and others advanced in the pipeline. However, with LV-GT patients reaching now >10 years of observations in the first treated patients, there is still limited information on the characteristics of long-term engrafted engineered HSPC. In this project we are comprehensively studying the functional properties, clonality and maintenance of stemness properties of LV transduced HSPC in long-term GT treated (LT-GT) patients with Wiskott-Aldrich Syndrome (WAS, n=6) and Metachromatic Leukodystrophy (MLD, n=10 HSC-GT known as atidarsagene autotemcel) reaching a follow-up of >8 years post-treatment.

Longitudinal analyses of bone marrow (BM) HSPC content in LT-GT patients (follow-up ranging from 2 to 11 years) show stable HSPC composition and count starting from 1-year post-GT, at levels comparable to pre-GT state in all the patients analyzed. Moreover, the composition of the most primitive HSPC remains stable over time, implying maintenance of the self-renewal/differentiation rate of primitive hematopoietic stem cells. Furthermore, we observed the preservation of a consistent transduced cell chimerism in the BM of LT-GT patients, measured through the frequencies of transduced cell colonies.

We are currently performing integration site (IS) retrieval on purified HSPC and mature lineages from LT-GT patients, collecting almost 45,000 IS from sorted HSPC in both MLD-GT and WAS-GT patients. We measured the clonal diversity of gene-corrected long-term engrafted HSPC through Shannon Index, and we found a stable and highly polyclonal pool of engineered HSPC in all the GT patients analyzed. Moreover, by assessing the probability of IS recapturing at different time points, we observe stable size of the HSPC pool over time in our cohort of LT-GT patients up to 10 years post-GT.

Finally, we investigated the maintenance of multi-lineage hematopoietic output in our cohort of GT patients. Specifically, we assessed the level of sharing of IS between HSPC and myeloid, lymphoid as well as erythroid cells, defining as multi-lineage the clones showing output toward all 3 compartments. Our data show that the vast majority of the engrafted HSPC have multi-lineage contribution, with similar proportion of clones found in the single compartments.

These data will be complemented with ongoing *in vitro* and *in vivo* functional studies and recently developed methods to retrieve multi-omic data at single-cell level, including transcriptome, chromatin accessibility, integration sites and mitochondrial DNA (mtDNA) diversity, allowing the collection of unprecedented information on the behavior of HSPC clones and subclones.

Overall, the information generated from these studies will increase our knowledge on human HSPC properties and on the capability of engineered HSPC to maintain a functional and safe long-term graft, ultimately validating the hypothesis of HSC-GT as a sustained, effective and life-long disease-modifying treatment and broadening its application to other genetic diseases.

P0413

Targeted suicide gene therapy: a novel approach for lung cancer treatment

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Lung cancer is one of the most pressing global health challenges. Its incidence and mortality rates have risen significantly, contributing to millions of deaths each year. While traditional treatments, such as chemotherapy and radiation, have shown some effectiveness, they are often accompanied by severe side effects and a lack of specificity, harming healthy tissues along with cancerous ones. This has led to a growing interest in innovative approaches like gene therapies. One particularly promising strategy is suicide gene therapy. This technique allows for targeted destruction of lung cancer cells by introducing genes that trigger selective cell death, sparing healthy tissues and reducing the need for additional toxic treatments. In the present work our purpose was to investigate the antitumor efficacy of *hokD* gene, from Escherichia coli k12, under the control of tumor-specific promoters in lung cancer cells versus non-tumor cells. Experiments were conducted in vitro in 2D and 3D culture model using EnSightTM Multimode Plate Reader and imaging techniques. Lung cancer CSC subpopulations were enriched by culturing primary and secondary spheroids in serum-free medium under anchorage-independent conditions, following the protocol outlined in patent WO 2016/020572 A1.Our results showed that *hokD* gene expression under the control of tumor-specific promoter causes a drastic inhibition of several lung cell lines proliferation in both 2D and 3D models. Moreover, an important decrease on CSCs viability was observed without any effects in non-tumor cells. Taking into account our results, this combination of gene and promoter could be a great option in future lung cancer therapies.

P0414

Cryopreserved Mesenchymal Stromal Cells overexpressing CXCR4 and IL10 maintain their enhanced therapeutic efficacy in a xenograft mouse model of graft-versus-host disease with preferential biodistribution to target organs

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Mesenchymal stromal cells (MSCs) are currently one of the cell type more frequently used in advanced therapies due to their unique properties. Previous preclinical studies have shown that MSCs can modulate graft-versus-host disease (GvHD) following allogeneic hematopoietic transplantation, although only moderate therapeutic effects have been observed in clinical trials. With the purpose of increasing the anti-GvHD effect of these cells, human adipose tissue-derived MSCs (Ad-MSCs) were transduced with a bicistronic lentiviral vector carrying the codon-optimized sequence of CXCR4 (coCXCR4), a molecule involved in cell migration to inflamed sites, and IL10 (coIL10), a cytokine with potent anti-inflammatory properties. In our previous work, we demonstrated that infusion of fresh genetically modified MSCs (CXCR4-IL10-MSCs) in a xenogeneic GvHD mouse model significantly reduced the severity of clinical and histologic signs of the disease compared to WT-MSC-treated mice. In addition, CXCR4-IL10-MSCs reduced the proliferation of pro-inflammatory Th1 and Th17 cells and induced the polarization of T cells toward an anti-inflammatory profile, as well as a significant increase in the number of regulatory B and T cells. The future availability of cell therapies to the public will depend on simple and rapid logistics, as well as a robust and reliable delivery process. Cryopreservation remains the cell therapy industry standard for cell storage and transport, but its impact on the efficacy of MSCs is not well understood. In our recent data, we have shown that cryopreservation of CXCR4-IL10 MSCs does not affect the immediate expression of CXCR4 and rapid secretion of IL10 upon thawing. In addition, no significant differences were observed in their immunomodulatory properties against T-cell proliferation in vitro, with an increased efficacy of CXCR4-IL10-MSCs compared to WT-MSCs. Furthermore, biodistribution assays performed with cryopreserved MSCs in a xenogeneic acute GvHD mouse model showed that CXCR4-IL10-MSCs had a preferential distribution to GvHD target organs compared to WT-MSCs with coIL10 expression detected in the lung, spleen, small intestine and liver 48 hours after cell infusion. Taken together, our new data strongly suggest that compared to fresh cells, cryopreserved MSCs ectopically expressing CXCR4 and IL10 maintain their enhanced efficacy for the treatment of acute GvHD and have a preferential distribution to GvHD target organs where significant levels of IL10 are delivered.

P0415

Base editing-mediated introduction of Hb G-Makassar variant in CD34+ cells from SCD patients ameliorates disease phenotype

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The evolution of gene therapy has opened up new avenues for treating sickle-cell disease (SCD). These include either gene addition by introducing anti-sickling, mutant β- or γ-globin genes to hinder HbS polymerization or genome editing approaches to reactivate the developmentally silenced fetal hemoglobin (HbF). However, the competition from the endogenous β^s-globin for incorporation into the hemoglobin tetramer cannot be addressed merely by expressing or

reactivating anti-sickling globins and this may limit the overall therapeutic efficacy. Moreover, both gene addition and genome editing raise safety concerns including potential induction of insertional mutagenesis and genomic translocations, respectively. Recently developed base editors (BE) offer a higher safety profile compared to traditional gene editing methods, by avoiding the induction of double-strand DNA breaks, thus minimizing the risks of both off-target- as well as on-target consequences. BEs can induce site-specific A>G conversions facilitating the in-situ conversion of the SCD pathogenic codon (valine, CAC) to a non-pathogenic variant of β-globin (alanine, CGC), forming the Hb G-Makassar (HBBG-Makassar). This conversion results in the elimination of HbS, consequently preventing hemoglobin polymerization and ameliorating SCD symptoms. The aim of this study was to develop an in vivo applicable tool for the introduction of the A>G G-Makassar mutation in SCD donor-derived CD34+ cells using a non-integrating HDAd5/35++ vector which expresses a highly efficient adenine base editor (ABE8e). To allow enrichment of edited cells after O^6BG/BCNU treatment, our vector contained also an $mgmt^{P140K}$ gene. Following 48-hour transduction with HDAd 5/35++ Makassar vector, SCD CD34+ cells were treated or not, with 50μM O^6BG & 35μM BCNU and seeded in erythroid differentiation (ED) and methylcellulose culture to assess cell growth, differentiation/maturation, morphology and clonogenic capacity. Functional assays included reactive oxygen species (ROS) levels and sickling assays with sodium metabisulfite. Editing levels were estimated using Next Generation Sequencing (NGS). The selection of transduced cells by O^6BG/BCNU significantly increased the cells' growth rate (fold expansion: 10±0.1 versus 4.81±0.21 control group, p=0.009), clonogenic capacity ($p<0.05$) and the A>G conversion rate that reached 95±2% on Day 11 of ED culture. The increased production of G-Makassar β-globin in edited cells led to significant reduction of oxidative stress, a hallmark of SCD (ROS-MFI in GlyA+ cells: edited 3783±242 vs control 12220±1729, p=0.008). G-Makassar β-globin expression also improved the erythroid cell differentiation and morphology manifested as significantly increased percentage of terminally differentiated erythroid cells (%GlyA+/NucRed- cells) (p=0.01) and decreased sickling of transduced/selected cells (% sickling: edited 19.74±3.59% vs control 48.95±3.44%, p=0.004). Overall, we present an alternative, effective method of precise base editing for SCD by indirectly repairing the causal mutation using an HD Ad5/35++-BE which can potentially overcome critical obstacles to clinical translation of gene editing for SCD.

P0416

CXCR4 drives hematopoietic stem/progenitor cell mobilization propensity upon G-CSF and Plerixafor administration in transplant donor or patients undergoing autologous gene therapy

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Mobilized Hematopoietic Stem/Progenitor Cells (mHSPC) are becoming the preferred cell source for autologous HSPC-gene therapy (GT) due to increased accessibility and number of collected cells with respect to bone marrow aspirates (BM). However, there is limited information on the composition and kinetics of responses of distinct HSPC subsets to the mobilizing agents Granulocyte Colony-Stimulating Factor (G-CSF, G) and C-X-C Chemokine receptor type 4 (CXCR4) antagonist Plerixafor (P), the two mostly used drugs for collecting HSPC in the context of GT trials. A better understanding of HSPC mobilization dynamics and mechanisms may allow improving HSPC mobilization strategies, thus the aim of this study is to assess the response of distinct HSPC subsets to G+P administration. We studied HSPC mobilization kinetics in 29 patients with different inherited disorders, including primary immunodeficiencies and lysosomal storage disorders, whose autologous mHSPC were collected for the purpose of gene therapy or backup, as well as in 16 adult healthy subjects who donated for allogeneic transplantation. By quantifying HSPC subsets in the BM and peripheral blood (PB) at steady state and in the PB after administration of G and G+P through high-resolution flow-cytometry, we found that Hematopoietic stem cells (HSC), multi-potent progenitors (MPP) and common myeloid progenitors (CMP) showed the highest mobilization capability after G, while all HSPC subpopulations are mobilized after G+P. Since both G and P act on CXCR4 retention axis, we hypothesized that the distinct response to G and P was the result of diverse CXCR4 expression pattern on HSPC subpopulations. Indeed, we measured differential CXCR4 expression and polarization among the distinct HSPC subpopulations in BM before mobilization, with HSC, MPP and CMP displaying lower CXCR4 expression and higher CXCR4 polarization over the other HSPC subsets. Moreover, CXCR4 expression inversely correlates with the subset-specific mobilization propensity. By reproducing patients' mobilization protocol in immunodeficient mice transplanted with human HSPC, we confirmed that the response to mobilizing agents of each HSPC subset depends on its CXCR4 expression. Indeed, CXCR4low HSPC were mobilized in the PB, while CXCR4high HSPC were retained in the murine BM during mobilization procedure. Finally, *in vitro* migration assays and *in vivo* homing experiments suggest that the expression and polarization state of CXCR4 impact on the migratory/homing features of HSPC subsets.

From a translational point of view, we measured comparable G+P mHSPC composition in all the patients analyzed suggesting similar response of HSPC subpopulations to G and P administrations, independently from the disease background. We also found that the number of primitive HSPC in PB after G+P correlated with their PB count before mobilization, identifying a possible predictive factor for the HSPC mobilization efficiency.

Overall, our results showed that CXCR4 expression plays a pivotal role both in driving HSPC mobilization propensity in response to G and P as well as the homing capability of HSPC subsets, providing clinically-relevant information on the design of improved mobilization procedures.

P0417

Unconventional design of mobilizer-resistant CXCR4 variants to enable chemotherapy-free conditioning in Hematopoietic Stem Cell Gene Therapy

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Hematopoietic stem/progenitor cell gene therapy (HSPC-GT) is emerging as a successful treatment for various primary immunodeficiencies, hematological, and storage diseases. This therapy involves mobilizing and harvesting HSPCs from the patient, genetically correcting them ex vivo through gene transfer or gene editing, and reinfusing them after administering partial or fully myeloablative conditioning to create space in the bone marrow for the modified cells. However, these conditioning regimens are associated with significant short- and long-term toxicities. Previously, we demonstrated that using combinations of mobilizing agents such as G-CSF, AMD3100, and BIO5192 creates a window of opportunity for the seamless engraftment of corrected cells. These mobilizers transiently deplete bone marrow niches, enabling replacement by the infused cells, especially if provided with a transient homing advantage, allowing a novel, toxic-free Mobilization-based HSPC transplantation (M-HSCT).

In the context of M-HSCT, understanding the kinetics of HSPC mobilization in human patients is crucial for the timely infusion of engineered HSPCs. Infusing these cells too early or too late may result in their failure to engraft due to re-occupied niches. Here, we explored strategies to increase the efficiency and prolong the engraftment window of engineered HSPCs. Specifically, we investigated the potential use of CXCR4 variants that are resistant to the antagonist AMD3100, while still retaining sensitivity to its chemotactic ligand CXCL12. In this strategy resident cells are mobilized by AMD3100, and replaced in their niche by engineered HSPCs that transiently express a CXCR4 variant that is resistant to AMD3100. We identified CXCR4 variants from the literature or generated them using directed evolution techniques and in-silico mutagenesis scanning through docking simulation.

Transient overexpression of these CXCR4 variants via mRNA led to increased surface expression of CXCR4 and endowed human HSPCs with partial resistance to CXCR4 antagonists *in vitro*. To evaluate the potential of these CXCR4 variants *in vivo*, we utilized a humanized mouse model. In this model, human HSPCs are first transplanted into mice to establish a human graft. The mice are then mobilized and infused with GFP-labeled cells from the same human donor, allowing us to distinguish newly infused HSPCs from previously engrafted, mobilized resident cells. Our results showed that transplantation of human HSCs transiently overexpressing a drug-resistant CXCR4 variant into mobilized hematochimeric mice resulted in enhanced engraftment compared to cells overexpressing wild-type CXCR4. Additionally, increasing the AMD3100 dose and prolonging its administration further improved the competitive advantage of the CXCR4 variant-expressing cells. We are now combining AMD3100-resistant CXCR4 variants with other engraftment enhancers to generate a potential synergistic effect.

Our results have the potential to transform HSCT into a safer, more widely applicable therapy, extending its benefits to a broader demographic and diverse healthcare settings.

P0418

Transient overexpression of four transcriptional regulators increase proportions of phenotypic CD34+ hematopoietic stem cells in *ex vivo* expanded hematopoietic progenitor cells

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CD34+ hematopoietic stem cells (HSCs) are rare cells in the bone marrow with the ability to self-renew and produce mature blood and immune cells throughout a human's life. These cells can be transplanted between individuals, replenishing the recipient's blood and immune system. HSCs are crucial in treating diseases of the hematopoietic system, but can be challenged by low numbers of long-term repopulating HSCs (LT-HSCs) or the loss of these during cell therapy manufacturing. To increase the total LT-HSC numbers in ex vivo cultures, we envisaged that CRISPR activation (CRISPRa) or mRNA delivery to transiently express key transcriptional regulators might de-differentiate CD34+ hematopoietic progenitor cells (HPCs) into LT-HSCs. We initially screened 34 genes for their susceptibility to CRISPRa by electroporation of the lymphoblast cell line, K562, with dCas9-VPR mRNA and sgRNAs targeting the transcriptional start sites of the genes. RT-qPCR analysis 24-hours post electroporation showed >10-fold upregulation of 18 of the genes and for the remaining 16 genes, mRNA encoding the open reading frame was produced by in vitro transcription as an alternative to CRISPRa. We clustered the candidate genes into eight groups and electroporated combinations of CRISPRa components and mRNA into 7-day pre-expanded CD34+ cells. By flow cytometry analysis, we screened for the LT-HSC-enriched surface marker phenotype CD34+/CD90+/CD45RA-/CD201+/CD49c+ 48 hours post electroporation. Two groups showed increased proportions of phenotypic LT-HSCs in CD34+ cells from mobilized peripheral blood and cord blood. Using a “minus-1-approach” we narrowed the candidates to two genes that combined gave a 3-fold increase in phenotypic LT-HSCs. Testing these two genes with 5 additional transcriptional LT-HSC regulators from the literature, we observed a 4-5 fold increase in phenotypic LT-HSCs. Again, using the “minus-1-approach”, we finally narrowed down the panel to 4 genes that gave the same effect. Interestingly, we observed an additive effect when the aryl hydrocarbon receptor antagonist, StemRegenin 1 (SR1), and CoREST-targeting small-molecule, UM171, were added to the media immediately following electroporation. This combination led to a significant increase in LT-HSC proportions from around 1% to 20%. We then tested these cells for long-term engraftment potential in sub-lethally irradiated female NOG mice but observed a substantial decrease in human engraftment. RNA-sequencing of the LT-HSC population confirmed upregulation of multiple LT-HSC signature genes, but revealed a significant decrease in CXCR4 expression that may negatively impact bone marrow homing following IV injections. Overall, our approach shows the potential of using RNA delivery of either CRISPRa reagents or open reading frame mRNA to transiently alter transcription levels of select genes in a multiplexed fashion to dramatically alter cell phenotypes. Such therapeutically relevant genetic engineering holds great potential to increase the LT-HSC pool after ex vivo expansion of CD34+ hematopoietic cells, but further refinements are needed to confirm true stem cell potential of the phenotypic LT-HSCs and increased in vivo engraftment.

P0419

Preclinical assessment for gene addition strategy in familial hemophagocytic lymphohistiocytosis related to Munc 13-4 deficiency

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Familial Hemophagocytic Lymphohistiocytosis (FHL) comprises a group of rare genetic disorders with defect cytotoxicity in T and NK cells predisposing to severe hyperinflammation with the clinical hallmark of the Hemophagocytic LymphoHistiocytosis (HLH) syndrome. The uncontrolled proliferation of activated T-CD8 lymphocytes and macrophages infiltrates lymphoid tissues, bone marrow, and multiple organs, including the brain. Thus, if untreated, full-scale FHL is usually fatal. FHL3 is due to mutations in the *UNC13D* gene coding for Munc 13-4 protein, one of the components of the perforin-dependent cytotoxicity apparatus. This condition accounts for 30% to 35 % of all genetic cases of HLH. Hematopoietic stem and progenitor cells (HSPCs) transplantation, which is the only curative treatment for FHL3 to date, remains difficult even when a compatible donor is available because of the patients' critical inflammatory background. In this context, gene therapy could be a promising therapeutic option, especially for those patients without HLA-compatible donors. The proof of concept of gene addition strategy for FHL3 has been previously demonstrated in mouse models. We then translated this approach to human cells using a third-generation self-inactivated lentivirus vector pseudo-typed with a conventional vesicular stomatitis virus-G (VSVG) expressing a codon-optimized UNC13D gene under the transcriptional control of EF1 alpha (LV-EF1a-UNC13D). Patient's cells were transduced with a GMP-like-grade LV-EF1a-UNC13D lentiviral vector. We studied the transduction efficacy from integration to restore cytotoxic function *in vitro*. Our GMP transduction protocol led to efficient transcription of optimized mRNA codon and Munc 13-4 protein expression as evidenced by RT qPCR and western blot, respectively. We also demonstrated the stability of the transgene integration after CD34+ differentiation toward CD3+ T-cells and NK cells with a vector copy number compatible with a clinical approach. Finally, biodistribution has been explored *in vitro* and *in vivo* and validated the absence of any toxic events. Altogether, our results support the feasibility and efficacy of UNCD13D gene transfer into Munc 13-4 deficient patient's cells. The clinical protocol has been submitted to the European Medicines Agency. Upon their approval, the first patients will be included in this phase I/II gene therapy clinical trial for Munc 13-4 deficiency.

P0420

Systematic evaluation and rational design of improved human adenovirus vectors for HSPCs and PBMCs transduction

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Over the recent years, a gene therapy conception for direct in vivo hematopoietic stem cell (HSPC) transduction was established. This approach combines mobilization of HSPCs from the bone marrow into the peripheral blood stream and intravenous injection of viral vectors for safe and efficient stem cell transduction. For direct in vivo transduction of HSPCs, a chimeric adenovirus type 5 (Ad5) vector carrying an optimized Ad35 fiber knob from species B (Ad5F35++) with increased CD46 binding was applied. However, a major limitation for the use of Ad5/35++ vectors in humans is preexisting serum antibodies against Ad5 that can neutralize intravenously injected vectors and prevent the transduction of target cells/tissues. Therefore, we explored a broader spectrum of human adenoviruses for HSPC transduction. In detail, nine alternative species B derived adenoviruses (Ad3, Ad7, Ad11, Ad14, Ad16, Ad21, Ad34, Ad35, Ad50), three

species D adenoviruses (Ad26, Ad37, Ad48), and Ad52 from species G were examined. Ad5 from species C and Ad5F35++ were included as controls. In vitro transduction with wild-type adenoviruses was first assessed in primary CD34+ HSPCs from three healthy donors by flow cytometry and qPCR. We found that Ad7, Ad11, Ad16, Ad34 and Ad35 have robust cellular entry efficiencies, evidenced by qPCR detection of viral genome and flow cytometry analysis of adenovirus hexon protein staining. Based on this information, we transformed these wild-type candidate viruses into replication-deficient E1-deleted first generation adenoviruses (fgAds) expressing a GFP reporter gene. The HSPC transduction with these GFP-expressing fgAds was analyzed via flow cytometry 48 hours post infection. All generated vectors showed efficient HSPC transduction, especially Ad11 achieved high transduction rates at low virus dose. In addition, to explore blood cells that can be relevant for in vivo immune therapy, we transduced peripheral blood mononuclear cells (PBMCs) from healthy donors with these vectors. We found that in contrast to Ad5, vectors based on Ad11, Ad16 and Ad35 showed significantly higher transduction efficiencies in monocytes. Interestingly, we observed that Ad16 reached up to 6-time higher transduction rates in NK-, B- and T-cells than the other analyzed adenovirus types, making it an interesting adenovirus serotype for immune therapy. Due to their large transgene capacity and improved safety profile, the helper dependent adenovirus (HDAdV) system is the best choice for direct in vivo delivery. Therefore we converted some promising adenovirus types (Ad7, Ad11, Ad16 and Ad34) to helper viruses (HV) for HDAdVs production. Given the minimal ITR sequence difference between Ad35 and other species B serotypes, we tested whether the generated helpers would be able to cross package the HDAdV genome with Ad35 ITRs. While HV34 can cross-package our previously produced HDAdV35 vector, the other HVs were less efficient in cross-packaging the HDAdV35 genome. This indicates the need for development of a serotype-specific HDAdV vector system. In summary, this study identified alternative species B human adenoviruses with efficient HSPCs and PBMCs transduction. The newly generated vectors hold high potential for in vivo HSPC gene therapy as well as direct transduction of blood lineage subsets.

P0421

Full pre-clinical and GMP-like scale-up study of CRISPR-based gene therapy aiming the ex vivo correction of the SCD mutation in HSPCs

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Sickle cell disease (SCD) is the most prevalent and clinically significant hereditary hemoglobin disorder in Brazil, affecting an estimated 60,000 to 100,000 individuals, and is caused by a single point mutation in the betaglobin (HBB) gene. Recently, the FDA approved the first CRISPR-based gene therapy for SCD, which uses gene editing to reactivate fetal hemoglobin (HbF) expression in autologous hematopoietic stem and progenitor cells (HSPCs). Several gene therapy approaches have shown promise for treating SCD, although only a few have focused on correcting the HBB gene. This latter approach would preserve natural gene expression regulation while eliminating the pathogenic sickle cell hemoglobin (HbS). Our group, therefore, performed a full pre-clinical and GMP-like scale-up study of CRISPR-based gene therapy aiming the ex vivo correction of the SCD mutation in HSPCs. High rates of ex vivo HBB gene correction were achieved through

homologous recombination (HDR > 40%) with CRISPR-Cas9 nuclease in combination with recombinant adeno-associated virus serotype 6 (rAAV6) carrying the donor template. We assessed the effects of increasing rAAV6 MOIs (312 to 1250) on gene targeting efficiency and determined their impact on HSPC function by performing colony-forming unit (CFU) assays and xenotransplantation in NBSGW mice. The frequency of corrected alleles in healthy donors' HSPCs increased with higher AAV6 vector genome titers, with no significant differences in their colony-forming ability. However, we observed a significant, dose-dependent decrease in hematopoietic reconstitution in NBSGW mice. In this regard, HDR levels above 20% in vivo were only seen in cells transduced with an rAAV6 MOI of 1250. Yet, this treatment resulted in the lowest cell chimerism after serial transplantation, suggesting that increasing doses of AAV6 negatively impact HSPC repopulation and differentiation potential. In parallel, we conducted scaling-up experiments using the G-Rex system at varying cell densities, comparing GMP-compliant media (AOF+HSA or CellGenix) to research use-only SFEM II. Over the first 48 hours of pre-stimulation, we observed no significant differences in cell differentiation, although GMP-compliant media resulted in a slightly higher frequency of mature cells. Of note, cells cultivated with AOF+HSA outperformed those with CellGenix in the CFU assay. HDR levels were statistically similar across all conditions 24 hours post-gene editing. However, CellGenix and SFEM II exhibited viability below 60%, whereas AOF+HSA maintained consistent viability around 70%. Cells from all these conditions were transplanted into NBSGW mice. In mock conditions, the engraftment rate exceeded 90%. In contrast, gene-edited cells displayed lower engraftment, which reflected in decreased numbers and diversity of hematopoietic cell in the periphery. Importantly, mice transplanted with AOF+HSA-cultured cells exhibited higher engraftment and immature cells frequencies in the bone marrow compared with those with CellGenix. In conclusion, the G-Rex system in combination with AOF+HSA media is suitable for GMP scaling-up manufacturing of gene-edited HSPCs. However, further improvements in the gene editing approach are required to reach relevant editing levels without compromising HSPC function.

P0423

Transduction Optimization of healthy donor HSCPs with a preclinical grade lentiviral vector for RPS19 Diamond-Blackfan anemia

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Diamond-Blackfan anemia (OMIM #105650) (DBA) is a rare bone marrow failure syndrome (BMFS) with a stimated prevalence of 5-7/1,000,000 per million live births. The clinical hallmark is erythroblastopenia, although congenital abnormalities and an increased incidence of cancer have been reported also in DBA patients. At least 40% of DBA patients are on transfusion support and no definitive therapeutic treatment other than HSCT is currently available for DBA patients under the age of 10. In older patients, these transplants are associated with high risk of toxicity,

resulting in a significant mortality and long-term morbidity, implying that there is an urgent unmet clinical need for a significant number of DBA patients. Most of DBA cases are associated with autosomal dominant mutations in any of 24 ribosomal protein genes, being RPS19 the most frequently mutated (25%). The preclinical studies conducted by CIEMAT/CIBERER/FJD provided evidence that an ex vivo lentiviral gene therapy approach to correct the genetic defect of RPS19-deficient cells with the developed PGK.CoRPS19.Wpre*-LV can constitute an efficient and safe approach to restore the hematological defects characteristic of RPS19-deficient DBA patients. These results have allowed the orphan drug designation “EMA/OD/0000060656” for this approach. Based on this, transduction with GMP-grade lentiviral particles manufactured by University of Bracelona/Creatio have been produced.

Optimization of transduction conditions and different MOIs in the presence and absence of transduction enhancers is being investigated in healthy fresh cord blood and mobilized peripheral blood CD34+ cells. We have tested the first pre-GMP grade lentiviral vectors in HSPC from healthy donors, optimizing the optimal dose of viral particles that we will need to obtain therapeutic efficacy, as well as functionality studies where we evaluate the safety of the lentiviral vector along with its correct expression inside the transduced cell. Furthermore, with the idea of optimizing transduction processes, we have evaluated different transduction enhancers, both individually and in combination.

Increased doses of virus concentration revealed, as expected, increasing levels of transduction that ranged from 83% to 97% both by analising the vector copies of the proviral sequency in the colony forming units and in hematopoietic progenitors maintained during 15 days in liquid culture. Analysis of vector copy number by qPCR ranged from 1.7 to 3.0 VCN/cell in the colony forming units demonstrating good transduction efficiency, compatible with a clinical application. Transduction optimizations are being carried out in order to reduce the amount of viral vector needed to achieve optimal transduction efficiencies in the presence of transduction enhancers previously described in other trial developed (NCT04437771 / NCT04105166 / NCT03825783) . Dose dependent expression of the transduction efficacy was observed.

P0424

Human RAC2 gain of function triggers inflammasome activation and patients’ immune deficiency revealing the RAC2-PAK1-NLRP3 pathway as a new therapeutic target

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A growing number of patients presenting severe combined immunodeficiencies associated with monoallelic RAC2 variants have been identified. Since the expression of the RHO GTPase RAC2 is restricted to the hematopoietic lineage and RAC2 variants contain single base pair mutations (e.g. G12R, Q61R, E62K, G12Vor A59S) this might be an excellent target for HSC-based gene editing approaches.

RAC2 variants have been described to cause immunodeficiencies associated with high frequency of infection, leukopenia and autoinflammatory features. Here we show that RAC2 activating mutations induce the NLRP3 inflammasome leading to the secretion of IL-1b and IL-18 from primary human macrophages. This induction depends on the RAC2 mutation and in particular on the associated RAC variant activation state.

Our data suggest that patients harboring RAC2 gain of function mutations might develop hyperinflammatory manifestations within a new type of inflammasome related disease. Furthermore, we show here the therapeutical potential of NLRP3 inhibitors or other inhibitors targeting the RAC-PAK-NLRP3 inflammasome pathway for symptomatic patients. However, inhibiting the RAC2 mutant triggered inflammasome activation might not be sufficient to cure these patients long term but may improve their quality of life until an appropriate donor for bone marrow transplantation has been found. To obtain a permanent cure, we are currently evaluating a CRISPR/Cas9 VLP-based (nanoblades) strategy to revert mutated RAC2 into its WT form at its endogenous locus.

P0425

Maximizing safety and efficacy in hematopoietic stem cell gene therapy

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Hematopoietic stem cell (HSC) gene therapy has been recently successfully employed for certain rare blood disorders including hemoglobinopathies, which resulted even in marketing authorizations such as Lyfgenia™ and Casgevy™. Despite these advancements, the appearance of leukemic events in the case of Sickle Cell Disease combined with the therapy's high price render the wider access, and hence the overall applicability of this approach, doubtful. Therefore to make HSC gene therapy more widely applicable, an effort is required toward robust and less laborious manufacturing processes that ensure reproducibility and predictability to maximize the safety as well as providing the capacity to generate engineered HSCs at large scale. Automation is the key to achieve these goals and, to this end, the CliniMACS Prodigy® as an automated, closed, GMP-compliant platform for HSC transduction is an ideal candidate to streamline cell processing. In this study, we intend to maximize the efficacy of $CD34^+$ cells viral transduction by testing several transduction enhancers to achieve a clinically translatable transduction efficiency both in the manual process (open steps) and in large scale on the CliniMACS Prodigy. Comparable cell recovery and viability between the small and large scale conditions were observed, however superior performance of this automated platform was repeatedly shown in terms of transduction efficiency compared to open manual steps. Moreover, significantly higher transduction efficiency is achieved in the presence of transduction enhancers, preserving cell recovery and viability, without altering the stem cell clonogenic capacity. Based on these results, we propose a

combined strategy of transduction enhancers on the automated platform to achieve significantly higher transduction efficiency. Ongoing experiments investigate the mechanisms involved in better performance of lentiviral transduction on the automated platform with molecular analysis via Next Generation Sequencing (NGS) and respective bioinformatics analysis.

P0426

Anti-tumour neutrophil polarisation via RIG-I pathway stimulation induced anti-tumour effects and immune checkpoint therapy resistance relief

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The tumour microenvironment harbours a variety of immunosuppressive cells, including certain neutrophil subtypes. Tumour-associated neutrophils (TANs) play widely distinct roles and can be broadly classified into anti-tumour (N1) neutrophils and tumour-associated immunosuppressive neutrophils, the latter can be further divided into pro-tumour (N2) neutrophils and granulocytic myeloid-derived suppressor cells (G-MDSCs), which suppress anti-tumour immunity and induce other immunosuppressive cells, complicating tumour treatment and potentially neutralising immune checkpoint inhibitors. Clinically, cancer patient mortality and recurrence rates correlate positively with N2 neutrophil counts in tumours, suggesting that neutrophils may have potential as cancer treatment targets. If naive or N2 TANs could be stimulated to polarise into N1 neutrophils, new treatments might emerge. However, TAN polarisation mechanisms remain elusive. Murine 4T1 mammary carcinoma is a mouse metastatic breast cancer model that is characterized by high neutrophil infiltration and also refractory to anti-PD-1 immune checkpoint inhibitor therapy. Polyinosinic:polycytidylic acid (poly i:c), a synthetic double-stranded RNA analog that shows promise as an adjuvant in cancer vaccines and immunotherapy. Poly I:C is known to interact with Toll-like receptor 3 (TLR3) when taken up passively by endocytosis, but when directly transfected into cells, poly I:C stimulates retinoic acid-inducible gene I (RIG-I) instead. In this study, we treated 4T1 tumour-bearing BALB/c mice intratumourally with poly I:C administered injected via electroporation (EP), an intracellular delivery method, or via classical needle-syringe (SI), a non-intracellular delivery method. We found that poly I:C EP treatment induced stronger tumour suppression effect and tumour-specific T cell-mediated immune response than via SI method. This EP-treated anti-tumour effect was suppressed by both RIG-I and TLR3 pathway inhibitors, while SI anti-tumour effect is dependent on TLR3 pathway only. Furthermore, both EP and SI treatments resulted in higher neutrophil infiltration into treated tumours, but only EP-treated tumours had higher proportion of N1 TANs. In addition, when depleted of neutrophils, EP-treated anti-tumour effect was abrogated, while SI-treated effect was enhanced. Interestingly, EP treatment could alleviate anti-PD-1 antibody therapy resistance in 4T1 tumour model, and this effect was blocked in the presence of RIG-I inhibitor. Taken together, our results showed that although poly I:C can stimulate both RIG-I and TLR3 pathways, intracellular delivery (EP) heavily favoured RIG-I pathway stimulation which promoted N1 polarisation in tumour-infiltrated neutrophils. These N1 neutrophils and induction of T cell-mediated immune response contribute in mounting an effective anti-tumour response. In contrast, non-intracellular delivery (SI) of poly I:C can only stimulate TLR3 pathway, facilitating neutrophil recruitment into tumour but without N1 polarisation. In conclusion, poly I:C treatment can induce different anti-tumour effects depending on the administration methods. RIG-I pathway stimulation can lead to N1 polarization in neutrophils and relieve anti-PD-1 therapy

resistance in 4T1 mammary tumour model. These findings may be useful in the consideration of potential applications of nucleic acid therapeutics in cancer therapy.

P0427

Influence of pre-existing inflammation on immune response to gene therapy in patients with Fabry disease

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Background:

Fabry disease (FD) is a lysosomal disorder characterized by a deficiency of the enzyme α-galactosidase A (α–Gal-A) due to mutations in the GLA gene, leading to the accumulation of the substrates globotriaosylceramide (Gb3) and globotriaosylsphingosine (lyso-Gb3) in multiple cell and tissue types, including the endothelial vessel wall causing immune activation. Consequently, inflammation becomes chronic, contributing to the permanent release of cytokines, which enhance the pathologic cycle and lead to organ damage.

The current FD therapies, recombinant α-Gal-A enzyme (Enzyme Replacement Therapy, ERT), and oral chaperone therapy while slowing the disease, there is still progression of FD with cardiac, renal, and cerebrovascular complications. There are currently multiple first-in-human gene transfer therapy (GTT) trials using adeno-associated virus (AAV) with the potential to replace ERT for continuous production of α-Gal-A in the liver. However, newly emerging data reveals that GTT might trigger an immune response, potentially leading not only to interference with the gene therapy itself but also to the development of immune-mediated complications, including microangiopathy. It is suggested that the changes in vascular compliance and the activation of chemo/cytokines and thrombotic factors are associated with vasculopathy in FD, raising the possibility that FD patients with chronic inflammation might experience a heightened immune response against AAV-GLA GTT.

Methods: In this study, we explored the impact of the proinflammatory status and complement activation in subjects with FD prior to and after GTT. We assessed six male patients with classic FD and varying genotypes, disease activity, and FD-specific biomarker levels. Plasma-EDTA specimens were collected (IRB-NCT02000310), and the Luminex Cytokine-96-Plex panel (Eve Technologies) was used to measure inflammatory activity in FD patients. Multiple variable methods, principal component analysis (PCA), and heatmap clustering analysis were performed to identify the cytokine secretion profiles.

Results: Among the four subjects who had received AAV-GLA GTT, D-dimer (3/ out of 3 analyzed), and lactate dehydrogenase, LDH (2/out of 4) were increased, and there was an elevation of total complement levels (CH50) and decreased C4Cc in one subject prior to dosing. The PCA and hierarchical analysis of the Cytokine-96-Plex panels from FD patients identified 5 clusters, with 4 clusters containing inflammatory/immune cytokines and one cluster representing interleukins and growth factors. Each patient displayed a unique cytokine response to the therapy indicating individual variations in the immune response. Additionally, it was observed that after GTT, the innate immune response played a crucial role in triggering the secretion of pro-inflammatory cytokines (Il-2, INF-γ, Il-3, Il-12p70, and MIP-1-α), which later normalized.

Furthermore, a decrease in several immune cell activators, such as RANTES, TNFα, CTACK, Eotaxin, or sFasl, were noted.

Conclusion: The findings of this study highlight the importance of closely monitoring the inflammatory status in patients with FD both before and after GTT. The involvement of the complement system and an increased cytokine response in some FD patients suggests that a personalized approach may be needed to select and monitor candidates for GTT, to prevent severe complications such as microangiopathy.

P0428

Development of mRNA structures and delivery systems for modulating immunogenicity

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Discovering approaches to modulate the immunostimulatory effects of in vitro transcribed mRNA (IVT mRNA) is crucial for the successful development of mRNA-based therapeutics/vaccines. The required balance between immune responses and translation may differ depending on the specific aims of clinical applications. In vaccine development, it is important to find appropriate levels of both translation and immune responses for vaccine efficacy. On the other hand, mRNA-based therapeutics such as protein replacement therapy primarily necessitate translation for sufficient protein expression without triggering any immune responses. In this regard, the primary goal of mRNA-based therapeutics/vaccines is to develop a range of strategies aimed at reducing the excessive immunostimulatory effects of IVT mRNA to varying degrees for different clinical applications.

We have innovated three key approaches to adjust the immunogenicity of mRNA-based therapeutics and vaccines. Firstly, we introduced the Additional Chimeric Element incorporated mRNA (ACE mRNA), a groundbreaking method for modulating immune reactions and amplifying the protein expression of IVT mRNAs. This technique involves embedding RNA/DNA chimeric elements into unmodified IVT mRNA, which significantly lowers type I IFN responses and boosts protein production. Secondly, we engineered a unique IVT mRNA capable of concurrently expressing and inhibiting target proteins, a combined hybrid structure of siRNA-tailed IVT mRNA (ChriST mRNA). The ChriST mRNA, a hybrid structure combining the functions of siRNA and IVT mRNA, enhances the immunogenicity of Ψ-modified IVT mRNAs by simultaneously expressing and suppressing target proteins. Lastly, to improve the efficacy of mRNA-based cancer vaccines, we incorporated an additional adjuvant (Pam3) into the mRNA delivery mechanism using lipid nanoparticles

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P0429

The dissociation constant is one of the limitations for cell-based neutralizing antibody assays against AAV capsids

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Gene therapy using adeno-associated virus (AAV) vector is currently in broad clinical applications. However, the presence of neutralizing antibody (NAb) against AAV capsids is known to significantly suppress its efficacy. Therefore, it is important to establish a highly sensitive assay to detect NAb. Current in vitro cell-based NAb assays may not be sufficiently sensitive to detect low-titer NAb due to the lack of sensitive cells for transduction, particularly for AAV5, AAV8 or AAV9. In this study, we designed to develop a sensitive assay for NAb against a variety of AAV serotypes, which utilizes commonly available cell lines such as HEK293 and Huh-7 cells.

In cell-based NAb assays, the sensitivity of NAb detection can be increased by reducing the amount of AAV reacting with anti-AAV antibodies. By combining a highly sensitive reporter gene secNanoLuc and glucose-mediated enhancement of gene expression, we confirmed to perform a cell-based NAb assay with significantly reduced AAV vector dose. The cell-based NAb assay against AAV5, AAV8, and AAV9 using human immunoglobulin and anti-AAV mouse monoclonal antibody showed that reducing the amount of AAV tended to improve the sensitivity of NAb detection. However, we found that reducing the amount of AAV to less than 100 vg/cell did not significantly improve the sensitivity of the cell-based NAb assay. The same trend was observed for AAV1 and AAV2, which are highly infectious to cells.

One of the reasons of this limitation may be explained by the dissociation constant (KD). The binding strength between an antigen and an antibody is expressed by the KD. When the amount of AAV decreases sufficiently, the amount of NAb that bind to AAV decreases and the detection level of NAb may reach the limitation. In this case the amount of NAb that bind to AAV correlate to the inverse of the KD value (Ab + Ag ⇆ AbAg [AbAg] / [Ab][Ag] = 1 / KD). In this study, NAb titer for each AAV serotype at 100 vg/cell tended to correlate with the inverse of the KD value of each anti-AAV mouse monoclonal antibody. These results indicate that 100 vg/cell of this cell-based NAb assay may be close to the detection limit of NAb. In this cell-based NAb assay, when the amount of vector is less than 100 vg/cell, the binding rate of antigen and antibody will decrease significantly, which may lead to the detection limit of NAb.

P0430

KJ103, a novel variant of an IgG-degrading enzyme changes neutralizing antibody titers against AAV2 and AAV8, which leads to rescuing and multiple-dosing of AAV-based gene therapies

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Adeno-associated virus (AAV) vectors are efficient and widely utilized delivery systems for treating gene-deficient diseases, with seven AAV-based gene therapies approved to date. KJ103, a novel IgG-degrading enzyme derived from non-pathogenic *Streptococcus equi subsp. equi*, has been identified as a potential pre-treatment to enhance AAV-based gene therapy by eliminating AAV-neutralizing antibodies (NAbs), thereby improving safety and efficacy, expanding the treatable population, and enabling re-dosing. NAbs against major AAV serotypes, prevalent in 30-70% of the population due to prior infections, usually trigger innate immune responses, inhibit viral transduction, and cause side effects, thus limiting the target population, treatment design, and re-dosing of AAV-based gene therapies.

A randomized Phase I trial conducted in China demonstrated that KJ103 rapidly cleaves IgG (95% within 6 hours post-administration) and maintains low IgG levels for one week, with levels gradually returning to baseline within one to two months. This study included five distinct dosage groups and reported no serious adverse events. Building on these Phase I results, an exploratory serum analysis was performed to assess changes in NAbs titers against AAV2 and AAV8 serotypes in healthy participants, as well as changes in F(ab')2 levels to provide a comprehensive view of the therapeutic window post-IgG cleavage. AAV2 and AAV8 were selected due to their high prevalence to maximize data from the limited Phase I participants.

NAbs titers were evaluated in 34 participants with detectable pre-existing NAbs before and after KJ103 administration on Days 0, 2, 3, 4, 7, 14, and 21. Titers decreased by more than 90% and were maintained for several days, indicating that KJ103 facilitates the administration of a second dose of AAV gene therapy. These findings suggest that KJ103 could effectively mitigate pre-existing NAbs, potentially broadening the therapeutic application of AAV gene therapies to a larger population.

P0431

Unequivocal detection of AAV-mediated gene doping: a two-step approach to the identification of vector transduction events

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Adeno-associated virus (AAV)-derived vectors are currently the leading platform for in vivo gene transfer in humans. Efficacy, safety, and long-term expression, that make of AAV an ideal vector for clinical use, may encourage the misuse of this gene delivery tool. Successful gene transfer with AAV vectors has provided tools and opportunities for genetic modification of functions that affect normal human traits, including athletic performance. Gene doping, defined by the World Anti-Doping Agency (WADA) as "the nontherapeutic use of cells, genes, genetic elements, or of the modulation of gene expression, having the capacity to improve athletic performance", is perceived as a coming threat and a prime concern to the antidoping community. Thus, there is an urgent need for novel strategies to detect the illegal use of AAV-based gene transfer. AAV vectors derive from a virus that naturally infects humans in childhood, consequently, a large part of the world population has anti-AAVs antibodies. Extensive surveys on the prevalence of anti-

AAV antibodies in humans have been published. Pre-existing immunity against AAV vectors represents the major challenge in the development of an antibody-based assay to discriminate natural infection from the use of AAV vector-based gene doping. However, anti-AAV vector antibodies are, potentially, a nearly perfect biomarker, easy to screen, stable at long-term post infusion and suitable to identify subjects likely to have used gene doping with AAV. Antibody subclasses analysis in healthy donors showed that anti-AAV IgG1 antibodies are highly prevalent in humans exposed to the wild-type virus, whereas no IgM are usually found in this population. Moreover, while immunosuppression given at the time of AAV treatment could reduce the cytotoxic immune response, clinical studies in patients undergoing AAV vector infusion suggest that anti-AAV antibodies raise anyway to high levels despite immunosuppression. Based on the hypothesis that gene transfer with AAV vectors leaves an immunological footprint clearly distinguishable from a naturally occurring AAV infection, we evaluated the serological profile of a cohort of healthy donors and AAV8 injected patients from a clinical trial. We developed a quick and stable method to test samples against ten rAAV at the same time. ELISA assays with the ten most frequently used capsids in human led to the identification of two promising biomarkers to distinguish between wild-type infected population and injected subjects. By comparing the levels of antibodies against the injected AAV to one of the most common in humans, we identified higher titers and serotype selectivity as biomarkers that can distinguish between natural infection and rAAV administration. The evaluation of IgG subclasses profile in AAV8-treated subjects surprisingly showed a significant presence of IgG3, not detectable in the healthy donors, suggesting its role as a specific biomarker for individuals that received an AAV8 vector. Our results mark a major progress in the detection of AAV-based gene doping by providing a valuable screening method and a more detailed characterization of the humoral response against AAV in humans. Further analysis on patients' sera receiving different AAVs by several routes of administration will allow to validate the robustness of the assay.

P0432

Death after intravenous rAAV9 gene therapy for acid ceramidase deficiency in a SMA-PME patient

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Spinal muscular atrophy with progressive myoclonic epilepsy (SMA-PME) is an ultra-rare lipid storage disease caused by biallelic variants in the *ASAH1* gene, which codes for lysosomal acid ceramidase (ACDase). Refractory myoclonic seizures and motoneuron dysfunction lead to respiratory complications and death. Currently, there is no cure for SMA-PME. GNT0009 is a recombinant adenovirus-associated viral 9 (rAAV9) vector containing the open reading frame of human *ASAH1* gene under the transcriptional control of the CAG promoter. Previous results showed that intravenous (IV) administration of GNT0009 in a severely affected mouse model of ACDase deficiency (*Asah1*$^{P361R/P361R}$) prolonged survival and corrected the phenotype at the

functional, histological, and molecular level. We present the case of a 15-year-old female affected with SMA-PME who carried 2 heterozygous *ASAH1* mutations. Deficient ACDase activity was confirmed on leukocytes and fibroblasts. Hearing loss, dyspraxia, ataxia, and myoclonus/absence epilepsy occurred between the age of 7 and 10. Peripheral neuropathy suggestive of motor neuron involvement was present whereas spinal cord and cerebral MRI were normal. Absence of peripheral organs and skin involvement ruled out Farber's disease. Subsequent decline in motor, executive and hearing functions led, at the age of 14, to loss of ambulation, assistance for dressing and feeding, total deafness and repeated myoclonic status epilepticus. A single IV injection of GNT0009 ($2.2x10^{14}$ vg/kg) was approved for compassionate use by the ANSM with the informed consent of the patient and her parents. At screening, cardiac, and respiratory functions were normal. Lamotrigine (400mg), clobazam (40mg), and perampanel (6mg) were administered daily. Prophylactic immune suppression included prednisolone and Sirolimus. Immunologic screening was negative. At day 3, the patient experienced fever, worsening myoclonia, inflammatory syndrome, and increase in liver enzymes and D-dimers. No anaemia or kidney injury was observed. A 20% reduction in baseline platelet count led to eculizumab prescription. At day 5, cardiac function rapidly deteriorated, progressing to cardiac shock and requiring high doses of catecholamines. Myocarditis was suspected. Glucocorticoids, tacrolimus, and veinoglobulines were inefficient. The patient received arterio-venous extracorporeal membrane oxygenation and haemodialysis. Interruption of care with immediate death was decided on day 8 due to generalised oedema, multi-organ failure and signs of brain injury. Elevated serum levels of INF-γ, MCP-1, MCSF, IP-10, and TNF were noted from day 2 to day 5, followed by a cytokine storm. A mixed picture of complement components was observed: SC5b-9 increased from day 4; CH50, complement factors C3 and C4 dropped on day 7. The consent for autopsy was obtained. Acute circulatory failure was the cause of death, with anasarca and shock-related injuries in the liver and kidneys. Few isolated ballooning neurons were observed in the medulla oblongata and spinal cord. There were no features of myocarditis, myopathy or thrombotic microangiopathy. Vector genomes were present at high levels in the liver, spleen, and lung, and at lower levels in different regions of the CNS. Administration of GNT0009 ($2.2x10^{14}$ vg/kg) in this SMA-PME patient induced an early refractory cardiac and circulatory failure related to a cytokine-mediated capillary leak syndrome five days after treatment.

P0433

Hydroxychloroquine for amelioration of mRNA and viral-vector gene therapy-induced inflammatory response

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Nucleic acid-based (NA) therapeutics become widely used through vaccination but are also applied for treating numerous genetic, cancerous, infectious, and degenerative diseases. Nevertheless, NA therapeutics are often highly reactogenic. Administration of NA therapeutics, such as mRNA, viral vectors or plasmids triggers innate immune response through activation of cellular NA sensing receptors, resulting in activation of type I interferon response. Inflammation is often desired to trigger activation of the innate immune system to provide a better response to

vaccination. On the other hand, a strong interferon type I response blocks mRNA translation, leading to its decay and limiting its therapeutic efficacy. Excessive inflammation may lead to pathologies, limiting the scope of NA therapies, therefore amelioration of strong innate immune response could increase the safety and widen the applicability of NA therapeutics.

We found that the introduction of mRNA via LNPs triggers a strong but transient inflammatory response, which peaks six hours after the application, determined in cell cultures and in mice. We reasoned that this transient response could be suppressed by the application of hydroxychloroquine (HCQ), which inhibits the activation of innate immune receptors through binding to nucleic acids. Indeed, prophylactic administration of HCQ or a concurrent application of HCQ and NA therapeutics strongly suppressed the production of proinflammatory cytokines. The strong protective effect of HCQ was demonstrated in vivo in the administration of adenovirus or mRNA delivered through lipid nanoparticles (LNP). Importantly, HCQ also suppressed the inflammatory response in LNP-delivered pseudomethyluridine-modified mRNA used in approved mRNA therapeutics, however, the suppression was even more potent for nonmodified mRNA. We suggest that HCQ protects the engagement of innate immune receptors to the introduced NAs in the period before they become protected by bound proteins and processing ribosomes. HCQ applied either before vaccination or mixed with mRNA/LNP vaccine suppressed the transient increase of inflammatory mediators without decreasing the translation and antibody titer, suggesting this formulation has substantially lower reactogenicity. Highly relevant for gene therapy, HCQ prevented damage to the brain-blood barrier triggered by the application of AAV9 to the brain, which supports the application of HCQ to increase the safety of gene therapy.

P0434

Myeloid cells associated with lung cancer influence the immune response to oncolytic adenovirus

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One of the appeals of oncolytic virotherapy is the capacity of viruses to change the tumor microenvironment, frequently interpreted as de-polarizing tumor-associated myeloid cells from highly pro-tumorigenic to less pro-tumorigenic phenotypes. This study aimed to analyze how therapy with oncolytic adenovirus AVID-317 modulates the complexity, phenotypes, and pro-tumorigenic function of tumor-associated myeloid cells.

We utilized single-cell RNAseq approaches and extensive complementary cytokine measurements in ex vivo cultures of primary human lung adenocarcinoma and a mouse model of orthotopic metastatic lung cancer. To explore the phenotypic and functional states of distinct myeloid cell populations before and after virotherapy, we performed large-scale correlation analysis to reveal cytokines and myeloid cells functional markers that play central roles in the tumor microenvironment after systemic virotherapy.

Our analyses showed that myeloid cells in lung tumors consist of complex cluster populations of macrophages, dendritic cells, and granulocytic cells in different polarization states. Oncolytic virotherapy led to increased expression of MCHII, CD86, CD80, and IDO1 on myeloid cells indicating activation of these populations. However, virus-mediated activation resulted in increased expression not only of type I IFN signature genes, and pro-inflammatory cytokines, but also potentiated expression of pro-tumorigenic genes and cytokines, such as PDGFB, IL4I1,

cathepsins, Lif, and IL-5. This simultaneous activation of M1 and M2 functional states of myeloid cells does not support the universal assumption of de-polarization of tumor-associated myeloid cells to lesser pro-tumorigenic phenotypes in response to oncolytic virotherapy. The clinical success of oncolytic virotherapy will require careful consideration of the TAM activation in response to virotherapy and finding effective drug combinations to suppress the pro-tumorigenic function of myeloid cells prior to administration of viruses with potent anti-tumor activity.

P0435

High-dose systemic delivery of an AAV9 vector carrying TLR9 immunomodulatory sequences results in improved gene transfer and limited hepatotoxicity in rats

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In recent years, recombinant adeno-associated viruses (AAVr) have demonstrated their effectiveness as gene therapy vectors for the treatment of genetic diseases. However, achieving a therapeutic effect with these vectors requires large doses, particularly when administered intravenously (IV). High-dose administration is nevertheless associated to toxicity, through the activation of the innate immune system. This is generally due to recognition of viral PAMPs (Pathogen Associated Molecular Patterns) by the patient's PRRs (pattern recognition receptors). In the case of rAAVs, one of the most reported PRRs is Toll-Like Receptor 9 (TLR9), which recognizes the non-methylated CpG motifs of viral DNA. This may lead to AAV toxicity and possibly initiate cellular and humoral adaptive responses against the viral capsid and/or the transgene product. One strategy for limiting AAV immunotoxicity is to block the TLR9 pathway. We therefore tested the impact of integrating a TLR9-inhibiting oligonucleotide sequence called DIMS (DNA Immunomodulating Sequence) in a rAAV9 viral vector on gene transfer efficacy and immunogenicity. Sprague-Dawley rats were intravenously injected with high doses (10^{14} vg/kg) of rAAV9 carrying the GFP gene alone or with the DIMS sequence. Three- and six-months post-dosing, rats were euthanized to analyse gene transfer efficiency in various tissues, monitor humoral and cellular immune responses and evaluate liver toxicity. DIMS sequence significantly increased transgene levels in most of the muscles, heart and brain tissues analysed. Moreover, this higher transduction was not associated to the presence of more immune infiltrates in muscles and hearts. However, at a peripheral level, the use of DIMS sequence did not decrease the cellular interferon-gamma response against the rAAV9 capsid. Regarding humoral immunity, a slight decrease in anti-GFP and anti-AAV9 antibody titers was observed. Because the liver is a major site for rAAV toxicity leading to a loss of gene transfer, we analysed the hepatic tissue. DIMS sequence enabled the maintenance of GFP expression in this organ as only 6% (1/5) rat no longer expressed GFP in the liver at 6 months post-injection, compared to 33% (2/6) in the rAAV9 control group. In conclusion, our data show that TLR9 immunomodulatory sequences can improve AAV-mediated gene transfer even in the case of systemic high doses, which make them a promising solution to optimize rAAV efficacy and safety.

P0436

Characterization of pre-existing anti-AAV cellular immune response using spectral flow cytometry in a cohort of healthy donors

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Recombinant adeno-associated viruses (rAAV) are efficient tools for in vivo gene transfer, with 7 FDA or EMA-approved drugs in the US and Europe. However, there are still major hurdles to overcome for their successful clinical translation such as their immunogenicity in patients. Indeed, adverse events related to immune system activation following systemic injection of high doses of rAAV in patients have been reported, resulting in some cases in clinical trial hold. This immunotoxicity is partly due to the reactivation of pre-existing T-cells specific for AAV capsid after gene transfer. We previously performed a cellular immune response prevalence study using the IFN-y ELISpot assay in a large cohort of healthy donors (n=145, Xicluna, Avenel et al., 2024). We showed that 24% and 46% of these donors have a pre-existing cellular immune response to AAV8 and AAV9 respectively. Here, we aim at better characterizing these anti-AAV8 and anti-AAV9 T cells (n=30 healthy donors per serotype). As the frequencies of these AAV-specific cells is very low, we developed an assay based on magnetic enrichment of AAV-specific IFN-y-secreting cells stimulated by peptides spanning the entire AAV capsid sequence. Using a staining combination of 24 surface markers, these cells are then analyzed by spectral flow cytometry. In addition to monocytes (CD16), NK cells (CD56), B cells (CD19) and T-cells (CD3/CD4/CD8) lineage markers, we are studying the frequencies of different CD4 and CD8 T cell subsets including effector-memory (TEM, CD3+CD45RA-CCR7-), central-memory (TCM, CD3+/CD45RA-/CCR7+) and effector memory re-expressing the CD45RA marker T cells (TEMRA, , CD3+/CD45RA+/CCR7-) as well as their migration and cytotoxic abilities using CX3CR1 and CD57 markers. Using the CD27/CD28 marker along with CD127, CD38 and HLA-DR expression marker, we are currently evaluating the activation and differentiation state of each subsets. We are also investigating the frequencies of anti-AAV regulatory T cells (CD3+/CD4+/CD25+/CD127-) and exhausted T cells (PD1+/TIM3+/LAG3+) that may be involved in immune tolerance to rAAV. Preliminary data on the 12 healthy donors analyzed (n=4 for AAV8 and n=8 for AAV9) showed the detection of AAV-specific TEMRA cells and CD8 T cells expressing exhaustion markers such as PD-1, TIM-3 and LAG-3 that are cell populations usually described in chronic viral infection. An in-depth characterization of these cells is currently ongoing. Finally, this study will allow to better characterize the preexisting anti-AAV T cells that might be involved in clinical trial adverse events and to develop targeted immunomodulation to prevent deleterious immune responses after rAAV gene transfer.

P0437

Seroprevalence of AAV-SLB101, AAV9, and AAVrh74 antibodies in a cohort of patients with Duchenne muscular dystrophy

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Adeno-associated virus (AAV) vectors of various serotypes are being used in gene therapy clinical trials for the treatment of Duchenne muscular dystrophy (DMD). The inclusion of patients for AAV gene therapy treatment is hindered by the presence of preexisting antibodies in a subset of patients which may lead to safety concerns as well as inhibit transduction. Thus, an understanding of AAV antibody seroprevalence and titers in DMD patients is important to help guide the development of strategies aiming to sufficiently reduce antibodies to facilitate safe and effective gene therapy treatment. Here, we report anti-AAV antibody data on our novel muscle-tropic capsid, AAV-SLB101, along with wild type capsids, AAV9, and AAVrh74. We measured neutralizing antibodies (NAb) using a transduction inhibition assay as well as total antibodies (TAb) using an ELISA (enzyme linked immunosorbent assay) assay that quantified IgG or IgM anti-AAV binding antibodies in serum from a cohort of 58 individuals with DMD. The samples evaluated were from donors aged ⩾2 to <19 years, with mean (std dev) age of 10 (4) years, and were obtained from CureDuchenne Link.

Using research assays, a significant correlation was observed between the NAb and TAb IgG levels in the seropositive DMD samples. Although the vectors belong to different clades, AAVrh74 (Clade E) and AAV9 (Clade F), cross-reactivity among the three serotypes was between 96-100%; 96% of patients who were seropositive towards AAV-SLB101 cross-reacted with AAVrh74, and 100% of patients who were seropositive towards AAV-SLB101 cross reacted with AAV9. Furthermore, we evaluated the seroprevalence of the 3 AAV serotypes across different ages. Highest seroprevalence was seen in samples from donors aged 7-9 years, followed by samples from donors aged 4-6 years. AAV-IgM titers were similar towards AAV9 and AAV-SLB101, however, no significant correlation was observed between TAb IgG vs TAb IgM levels. Approximately 50% of the seropositive donors had TAb titers of >1:6,400 (highest titer tested); towards AAVrh74 (58.33%), AAV9 (52.17%), and AAV-SLB101 (43.47%). Additionally, ~25% of seropositive donors aged 4-7 years had titers >1:6,400 across all three serotypes.

In conclusion, results from this study suggest that antibodies towards AAV9 and AAV-SLB101 are highly cross reactive and that there is a significant correlation between TAb IgG and NAb titers. Additionally, of the seropositive DMD donors, 50% have high anti-AAV titers of >1:6,400 among which 25% are aged 4-7 years.

P0438

Challenges in developing and validating companion diagnostic assays for gene therapy

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Anti-AAV antibodies may interfere with the effectiveness of AAV-based gene therapies (GTs) or trigger serious adverse immune reactions. Patients with pre-existing AAV antibodies are therefore excluded from most clinical trials evaluating GTs, ideally using a clinically relevant cut-off. Since AAV antibodies are not associated with the disease being treated, but rather with the AAV capsid of the GT, a clinically relevant cut-off can only be determined performing interventional clinical studies and not in natural history or epidemiological studies, as is the case for other endogenous diagnostics such as blood glucose or HDL. However, the diagnostic regulations of some countries that are relevant for the validation of patient selection assays complicate or even prevent the determination of such clinically relevant cut-offs limiting access of patients with higher pre-existing AAV antibody titers to a beneficial GT. This talk explains whether and under what conditions patients should be excluded from clinical studies, when a CDx is required, which regulations are relevant for CDx validation and why new regulations for the validation of AAV antibody assays used for patient selection are needed.

P0439

Harnessing pre-existing immunity to AAV for enhanced capsid engineering in safe and effective therapy

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We have initiated a comprehensive investigation into the immunogenicity risks associated with the use of adeno-associated virus (AAV) based recombinant vectors in gene therapies, which have witnessed an upsurge in their application for treating various debilitation diseases like monogenic, metabolic, and neuro-muscular genetic disorders. The purpose of our research is to gain a deeper understanding of the adaptive immune responses elicited by these vectors. In fact, the broad application of rAAV-based-gene therapy is limited by pre-existing immune responses induced by the natural exposure to wild type AAVs. For instance, the activation of T cells can result in the rejection of transduced cells, while B cells can produce anti-AAV neutralizing antibodies, which may directly bind the capsids thus inhibiting the transduction of target cells and the effectiveness of therapy. We have produced a comprehensive dataset on binding titers against AAVs in 900 healthy donors coming from several cohorts. Our primary findings confirm that the prevalence of pre-existing humoral immunity to WT viral vectors varies across capsid serotypes, age and geographical location. Additionally, using our human B cell platform, we have successfully isolated three broadly reactive anti-AAV monoclonal antibodies and determined their binding epitopes thorough CryoEM. This structural information has been utilized to guide capsid engineering, aiming at the generation of novel AAV variants with reduced response to neutralizing antibodies, while keeping high productivity and cell transduction ability. Alongside pre-existing immunity, the *de novo* host immune responses (innate and adaptive) can also determine the safety and effective treatment if a second administration is required. Therefore, we believe that our work and efforts can contribute to a comprehensive understanding of the mechanisms driving AAV vector immunogenicity in humans, with the goal of developing safe and effective AAV variants that can be used to treat patients with pre-existing immune responses.

P0440

Ex vivo investigation of AAV vector-specific adaptive immunogenicity in healthy volunteers

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Spinal muscular Atrophy (SMA) is a complex monogenic inherited motor neuron disease, affecting the survival motor neuron (SMN1) gene. In severe cases, SMN protein deficiency leads to motor neuron death, progressive loss of muscle control, and death before the age of two. Zolgensma approved gene therapy for SMA, consists of a recombinant AAV9 vector carrying a replacement wild-type copy of the SMN1 gene, producing functional SMN protein significantly improving neuronal and muscular function. However, Zolgensma treatment is associated with increased liver transaminases indicative of a cytotoxic T-cell response and therapy failure (EPAR MA 3.8.1). It is important therefore to develop assays to identify patients susceptible to AAV specific adaptive immune activation to prevent/manage hepatotoxicity in the clinic.

To identify the distribution of natural infection with AAV, serum or plasma samples from 124 healthy volunteers were interrogated for pre-existing AAV humoral immunity in the blood by ELISA. AAV9+ donors (n=37) showed cross-reactivity with AAV2+ of 86.6%, while only 40.2% of the AAV2+ donors (n=49) displayed cross-reactivity with AAV9. 26.1% of the AAV2 seropositive donors (n=33) could exhibit humoral or cellular immune responses against another AAV serotype, such as Zolgensma, yet would not be detected via AAV9 Antibody screening.

PBMC from selected volunteers were then exposed to AAV VP1 peptide pools over 24 hours and multiplex cytokine secretion array (n=7) was performed in parallel to intracellular staining (n=22), to 1) optimise a panel of cytokine markers best suited to detect AAV specific T-cell activation, and 2) identify specific T-cell populations (CD4, CD8, CD45RO) activated by AAV VP1 peptides, including MAIT cells (CD3+/TCRVa7.2+/CD161^{high}) an abundant T-cell population in the liver. In at least 4 donors an increase of TNF-α, IL-6, IL-10, granzyme B, perforin and IFN-y were seen. IL-6 expression was increased up to 10000 pg/ml in some healthy donors following PBMC peptide treatment.

ICS revealed increased IL-10 expression across all tested T-cell types (0.34% - 1.27% of T-cells). Increased expression of IL-6 was seen mainly in AAV2, while perforin showed a consistent increase in AAV2+AAV9+ donors after AAV2 and AAV9 exposure (up to 5% of T-cells). MAIT cells in healthy donor PBMC (n=3) showed an increase of expression in IFN-y (0.1-0.15%), and IL-17a (0.5%-1%), however, TNF-α expression was increased on average by 5.4% in the presence of AAV9 peptides and 1 % with the AAV9 mcherry vector.

Collectively these data demonstrate the presence of pre-existing immunogenicity towards the AAV VP1 protein in certain healthy volunteers. Further research is required to explore how this translates into adverse events in patients exposed to Zolgensma.

P0441

Depletion of AAV8 antibodies in seropositive healthy subjects and patients treated with the IgG cleaving enzyme imlifidase

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Pre-existing antibodies towards adeno associated virus (AAV) based gene therapy are a major challenge as they are present to a high extent in patients who are candidates for gene therapy, thereby excluding them from successful treatment and potential cure. In this study, we have screened serum samples from healthy subjects and from kidney transplantation patients, treated with the IgG cleaving cysteine protease imlifidase in clinical studies. Using a total AAV8 antibody (TAb) assay, samples from 36 individuals, of which a majority were treated with with one or two doses of 0.25 mg imlifidase/kg body weight, were screened. Out of the analysed individuals, approximately 15% were seropositive. For all seropositive individuals, pre-dose samples (0h) as well as several post dosing samples (time points 6h, 24h, 48h and 7days) were further analysed with an AAV8 TAb assay. For all subjects, a dramatic decrease in AAV8 antibody levels were observed as early as 6 hours after dosing with imlifidase. These results were expected, and quick and efficient antibody removal has previously been confirmed in other indications, including kidney transplantation, anti-GBM disease and Guillain-Barré syndrome, as well as *in vitro* with various antibodies, including donor specific antibodies, antibodies towards human leukocyte antigen (HLA) and antibodies towards different vaccines. More than 99% of all IgG is efficiently cleaved within hours after imlifidase dosing. Newly synthesised IgG starts to show after 5-7 days, thus creating an antibody-free window of approximately one week. IgG levels are normally restored to their initial levels within 6 months. The results from this small *in vitro* study on serum from imlifidase treated individuals, supports the rational for further clinical studies, as antibody depletion with imlifidase could offer a simple and elegant way to enable gene therapy in currently ineligible patient groups. The potential of imlifidase in removing AAV antibodies has previously been demonstrated in both *in vivo* and *in vitro* studies and is under further evaluation in collaboration with our gene therapy company partners.

P0442

Development of a novel nanoliter scale total antibody (TAb) assay to detect pre-existing antibodies against AAV vectors regardless of serotype

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In clinical trials with adeno-associated virus (AAV) vectors, screening for participants lacking pre-existing neutralizing antibodies is crucial for effective gene therapies. Similar to humans, non-human primates (NHPs) serve as natural hosts for AAVs and frequently harbour neutralizing antibodies against various AAV serotypes commonly employed in (pre-)clinical investigations. Therefore, pre-screening NHP cohorts for their antibody status is essential in gene therapy

studies prior to vector administration. Various methods, such as Neutralizing Antibody (NAb) assays and Total Antibody (TAb) assays, are used to screen for anti-AAV antibodies. NAb assays assess how NAbs and other non-antibody factors hinder AAV transduction while Tab assays, with their simpler design and operational ease, typically require serotype-specific assays or AAV capsid labeling (e.g bridging format), introducing time and variability. We developed a generic anti-AAV TAb immunoassay on the automated Gyrolab platform that enables the detection of anti-AAV antibodies in human and cynomolgus monkey samples, eliminating the need for serotype-specific assays or AAV capsid labeling. Genericity, i.e. the ability of the assay to accurately detect AAV vectors regardless of serotype, was assessed by testing for pre-existing TAbs against seven of the most commonly used AAV serotypes.

Anti-AAV TAb analysis in an automated, CD-based format was performed on the Gyrolab platform with commercially available Gyrolab Generic Anti-AAV kit reagents.

In the automated microfluidic method, biotinylated capture reagent is introduced into a microstructure in the Gyrolab Bioaffy 1000 CD and captured on streptavidin-coated beads in the flow-through affinity column. The second capture reagent, an AAV capsid of choice, is introduced and interacts with the first capture reagent forming a complex. Subsequently, samples are introduced into the microstructures where anti-AAV antibodies are captured by the secondary capture reagent. Finally, a fluorescently-labeled detection reagent is added. The integrated laser-induced fluorescent signal represents the response from the bound anti-AAV antibody to the AAV capsid.

Genericity of the assay was assessed by testing for pre-existing TAbs against seven AAV serotypes (AAV1, AAV2, AAV3, AAV5, AAV6, AAV8 and AAV9) for two sample cohorts: 30 human serum samples and 14 cynomolgus monkey serum samples: In the human cohort, 4/30 samples exhibited indiscriminate positivity for all tested serotypes, while 15/30 samples showed indiscriminate negativity. AAV1, AAV2, and AAV3 had the highest number of positive individuals, whereas AAV5 and AAV9 had the lowest. These findings align with established data on the prevalence of AAV serotypes in humans.

For the cynomolgus monkey cohort, 1/14 samples were indiscriminately positive for all serotypes, while 1/14 samples were indiscriminately negative. AAV8 and AAV9 had the greatest number of positive individuals. This finding aligns with existing data regarding the prevalence of AAV serotypes in non-human primates.

The distribution of positive qualification for various serotypes among different individuals, without uniform readings for all serotypes in all samples, suggests that the assay is indeed generic and capable of successfully discriminating between serotypes.

P0443

Elucidating Complement Activation Mechanisms in AAV Gene Therapy: Development of a Novel ELISA-Based Assay for Enhanced Safety and Efficacy

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Gene therapy utilizing adeno-associated virus (AAV) vectors demonstrates remarkable clinical efficacy in treating diverse diseases. Despite its relative safety, evidence from clinical trials has shown that AAV can trigger severe life-threatening inflammatory responses, especially in high-dose, systemic, administration. Many of these adverse reactions are associated with the secondary activation of the complement system, leading to complications such as acute kidney injury due to aHUS-like complement activation, thrombotic microangiopathy, thrombocytopenia, and immune-mediated myocardial inflammation. Though AAV-mediated complement activation is a major safety concern, we still lack understanding of the activation mechanism and a predictive method to prevent or mitigate activation in administered patients.

Our primary research focus has been to develop and evaluate an ELISA-based assay for assessment of AAV-mediated Complement Activation. This novel assay set-up involves combining an AAV-coated plate with a tailored buffer composition, enabling the assessment of complement system activation through the classical, lectin, or alternative pathways. While the platform is primarily designed to assess the potential of anti-AAV antibodies to initiate the classical pathway, across different serotypes of AAV, it inherently could detect general complement activation and dysregulation.

Evaluation of a small cohort revealed the novel assay's ability to provide additional information in detecting propensity for immunoglobulin-induced immune responses compared to a Total anti-AAV antibody (TAb) assay. Additionally, the exploratory investigation has indicated that the assay may also be used to screen the susceptibility for complement system overactivation.
Given the complexity of serum interactions with anti-AAV antibodies and the complement system, our assay shows promise in elucidating pivotal immune interactions and offers valuable insights to enhance the safety and efficacy of AAV-based gene therapies, thus, advancing the field.

P0444

Blockade of THE CD28/B7 costimulatory pathway with Abatacept inhibits immune responses to AAV vector and transgene product

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The immune response elicited to AAV vector capsids represents a significant obstacle to rAAV-mediated gene therapies, as neutralizing antibodies (NAbs) preclude effective redosing and can mediate adverse events in clinical trials. This is particularly problematic for patients treated early in life, who may require subsequent administrations. Moreover, cytotoxic T lymphocyte (CTL) responses against the rAAV vector or the transgene product can result in toxicities and the elimination of transduced cells. It is therefore of high interest to identify immunomodulatory strategies for preventing both humoral and cellular immune responses to AAV-mediated gene therapy.

Costimulatory signals by activated antigen-presenting cells are crucial for T cell activation, and costimulation blockade may induce T cell anergy or enhance the function of regulatory T cells, fostering tolerance. We first evaluated whether transient treatment with abatacept (CTLA-4-Ig fusion protein, blocking CD28/B7 interaction) could inhibit the formation of NAbs against AAV8

capsids following intravenous administration in mice, thereby facilitating redosing. C57BL/6 mice received an initial dose of AAV8-hSEAP (1E12 vg/kg, i.v.) on day 1, while a control group remained untreated. The experimental group received 10 mg/kg abatacept (OrenciaTM) i.p. every 2 days for one week, starting 2 days before AAV8-hSEAP injection. Redosing with AAV8-hFIX (5E13 vg/kg) was performed on day 22 in the absence of immunosuppressive treatment. Transgene expression and anti-AAV IgM and IgG titers were monitored over time. We found that while redosing was inefficient in mice that did not receive abatacept upon first AAV administration, transient treatment with abatacept effectively suppressed the formation of anti-AAV8 IgG antibodies, thereby permitting successful transduction by the second rAAV8 vector and subsequent expression of the transgene product hFIX at level equivalent to the control group.

In another experiment, we investigated the effects of abatacept on the cellular and humoral responses to the ovalbumin (OVA) transgene product in C57BL/6 mice injected intramuscularly with 1E10 vg of a rAAV1 encoding OVA. Treatment with abatacept for 14 days starting 2 days before gene transfer fully suppressed the induction of anti-OVA CD8+ T cells, which was still observed 8 weeks after rAAV-OVA dosing. In addition, abatacept drastically reduced anti-OVA IgG levels. This study shows for the first time that abatacept can reduce the cellular and humoral immune responses to the transgene product.

These results underscore the potential of costimulation blockade in enhancing the clinical applicability of rAAV-mediated gene therapy, by preventing the immune responses to both the AAV capsid and transgene product and enabling vector redosing.

P0445

Comprehensive Immunogenicity Analysis of AAV Therapies: Correlating Anti-AAV Antibodies (NAbs/TAbs) with Complement Activation Dynamics

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The extensive study of adeno-associated virus (AAV) vectors as carriers in gene therapy has progressed the field from a promising concept to a tangible medical reality. Despite these advancements, significant bottlenecks persist in the development of gene therapy products, particularly in meeting stringent regulatory requirements. A key challenge is the influence of pre-existing antibodies against AAV on the efficacy of *in vivo* AAV-mediated gene therapy. This includes issues like capsid neutralization and triggering of immune reactions, notably the activation of the complement system.

Currently, we lack a standardized testing regime, but analysis of both the presence of total binding antibodies (TAbs) as well as neutralizing antibodies (NAbs) has been recommended by industry leaders.). Additionally, assessing patient sensitivity to complement activation due to AAV adds a critical dimension to immunogenicity evaluation.

Here we present the evaluation on 100+ samples of Svar's AAV immunogenicity solutions, which includes the assessment of anti-AAV antibodies (both NAbs and TAbs) and each sample's sensitivity to AAV-mediated complement activation. Our approach first employs a novel cell-based iLite® AAV platform for accurate NAb assessment to detect NAb-mediated reduction of

AAV transduction. Secondly, TAbs are assessed with a standardized immunoassay with a high sensitivity, allowing the evaluation of the presence of anti-AAV antibodies Finally, the potential for AAV-mediated complement activation is evaluated in each sample, a crucial step in identifying susceptibilities that may lead to adverse effects. This evaluation is adaptable for both pre- and post-AAV administration scenarios.

These three assays combine the advantages of both immuno- and cell-based assays. The integration of these three assays is essential not only to prevent treatment failure but also to potentially avoid serious adverse effects such as complement activation. Moreover, this approach offers reliable and versatile, customizable solutions for assessment and differentiation of humoral antibody responses against AAV vectors of any serotype, thus addressing the expert demand for such assays. Svar's AAV immunogenicity solutions represent a unique toolbox designed to accelerate and support the clinical development of todays and tomorrow's AAV-mediated gene therapies.

P0446

Enhanced novel dual cell-based platform for Detection of AAV Neutralizing Antibodies with *iLite*® technology

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The extensive study of AAV vectors as carriers of gene therapy products has advanced the field from a promising new therapeutic concept into today's medical reality. However, several bottlenecks are still present during the development of gene therapy products; hence, meeting respective regulatory requirements has turned into a challenging process. The use of bioassays facilitates the progression through the different developmental phases as they can address key parameters for regulatory acceptance, including vector potency or determination of neutralizing antibodies (NAbs).

We present here the *iLite*® NAb AAV Platform, a novel two-component system for detecting and quantifying NAbs against recombinant AAV vectors. By employing ready-to-use reporter cells in a frozen format, it eliminates the need for continuous cell culture and specialized equipment. This approach enhances reproducibility and significantly reduces the time required to run the assay, completing the process in half the time of current alternatives.

Additionally, the iLite® AAV Platform incorporates an internal control reporter within the cells, designed to detect serum matrix effects that can result in false positive samples. This feature enhances the accuracy of the assays by allowing differentiation between true NAbs presence and artifacts caused by matrix interferences.

The assays presented here combined the features of a cell-based assay that can quantify neutralizing anti-AAV antibodies with the robustness and ease of an immune-detection assay, resulting in an ideal tool for AAV NAb detection.

P0447

Template plasmids for mRNA production – focus on poly(A) elements

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The production of mRNA for vaccine or for non-viral gene therapy applications has come into focus. Plasmid DNA is used as an effective DNA template for an *in vitro* transcription process based on T7 RNA polymerase. Enzymatic post-transcriptional 3'-polyadenylic acid sequence addition is often followed although it brings with it certain drawbacks such as an additional process step fraught with loss of yield and quality, and more importantly a reduction of mRNA product homogeneity due to variable poly(A) lengths. Many manufacturers find it attractive to have the poly(A) sequence encoded in the DNA template. This saves one downstream step and also ensures that poly(A) homogeneity is guaranteed. Maintaining such unnatural long homopolymeric repeats in plasmids during propagation in bacteria has been a focal point in our R&D labs. In this work, we present a comprehensive analysis of our successes in maintaining poly(A) stretches in the plasmids we have produced in the past. We also report on the creation of a universal poly(A) containing template plasmid that acts as a platform molecule to quickly take in any customer sequence of interest containing typically a T7 promoter, 5' UTR, CDS, 3'UTR. This is followed by the poly(A) stretch on the template as well as a recognition sequence for a Type IIs restriction enzyme to introduce a staggered cut at the end of and within the poly(A) sequence for linearization. This makes it a very practical template for *in vitro* transcription resulting in clean and homogeneous mRNA products. The removal of open circular forms of the plasmid during our purification ensures that no plasmid with a potential single strand break within the CDS would be present in the final product which is another step to avoid the generation of truncated mRNAs. The routine and reliable analysis of poly(A) lengths goes hand in hand with the work described above. Our optimized polyacrylamide gel electrophoresis protocols give a coherent and reliable snapshot of the poly(A) length in a plasmid even at the stage of clone screening. Furthermore, we have optimized our Capillary Gel Electrophoresis and recently also our High Performance Liquid Chromatography analysis methods to follow and characterize poly(A) lengths.

P0448

Clinical translation of CRISPR-Cas9-mediated TCR engineering for the treatment of viral infections

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The transfer of naturally occurring, virus-specific T cells has proven effective in treating a potentially fatal reactivation of latent viruses such as Cytomegalovirus (CMV) in immunocompromised patients (e.g., after stem cell transplantation). However, finding suitable seropositive donors is often challenging. Moreover, this approach is not an option in the case of seronegative donors. In contrast, antigen-specific TCR-engineered T cells allow for the development of adaptable 'living therapeutics', also for the high-risk setting of CMV^{+} recipient/CMV^{-} donor. We recently devised a CRISPR-Cas9-mediated T-cell receptor (TCR) delivery technique termed 'orthotopic TCR replacement' (OTR), which facilitates the generation of near-to-physiological engineered T cells with highly predictable functional properties.

We isolated several TCRs specific for an immunodominant HLA-A*02:01-restricted epitope derived from the CMV pp65 antigen via pMHC multimer staining using CMV seropositive individuals. TCRs were engineered into both primary T cells and Jurkat-based reporter cell lines to characterize the peptide sensitivity and cytotoxic capacity of OTR-T cells. Cytotoxicity was evaluated in an advanced model for endogenous epitope presentation. Based on these data, a lead candidate for clinical translation has been selected. In parallel, we translated the OTR approach into a GMP-compliant T-cell manufacturing process by designing and optimizing a 5-day process.

Currently, we are devising and compiling all necessary steps to start a first-in-human phase I clinical trial, which will encompass the prophylactic infusion of CMV-specific OTR-engineered T cells for stem cell transplant recipients with seronegative donors and high-risk of CMV infections.

P0449

Development Of Novel And Robust LNP-RNA Vaccines

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mRNA vaccines represent a novel and clinically validated approach to provide rapid responses with scalable solutions to ongoing and future diseases. Lipid nanoparticles (LNP) have enabled success of mRNA-based vaccines by providing an appropriate carrier and incorporating adjuvant features, such as innate immune activation, potent antibody production, and T cell responses to mRNA-LNP vaccines. This has been demonstrated in the historically successful clinical trials run by Pfizer/BioNTech and Moderna. Critically, the approved formulations represent repurposed formulations that were initially designed for hepatocyte gene silencing. These formulations in turn resulted in successful vaccines that were rapidly translated to the clinic for use in the COVID-19 pandemic. We aimed to develop fit-for-purpose mRNA vaccines to achieve robust immune responses. Here, we demonstrate that our novel ionizable lipids and proprietary LNP formulations can stimulate comparable and/or improved immune responses, such as robust T cell responses and neutralizing antibody production, to clinically approved mRNA-LNP vaccines.

Our LNP formulations consisting of an ionizable lipid, helper lipid, cholesterol, and PEG-DMG at differing molar ratios containing mRNA encoding for the SARS-CoV-2 Spike protein were used to screen our formulation library for an enhanced immune response. The mRNA-LNPs were

delivered intramuscularly into mice at days 0 and 21 at a low dose of 1 microgram. Innate immune responses were measured using Mesoscale Discovery (MSD) platform and T cell responses were measured using ELISPOT and Intracellular cytokine staining assay. Serum SARS-CoV-2 Spike IgG and neutralizing antibodies against the receptor binding domain (RBD) were measured using the MSD platform.

Through screening of our LNP library, we found that several of our proprietary ionizable lipids and formulations resulted in similar or enhanced T and B cell responses compared to clinically approved lipids and formulations. Specifically, NanoVation proprietary long circulating LNPs (lcLNPs) demonstrated comparable and/or improved immune responses at a low dose of 1 microgram when compared to clinically approved benchmark formulations.

Our ability to develop lipids and tailor formulations to specific needs provides additional tools to develop potent and effective vaccines for infectious diseases beyond SARS-CoV-2.

P0450

Mucosal delivery of mRNA-lipid nanoparticles using mucoadhesive films

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Nucleic acid-encoded therapeutics are programmable drugs that can be used for therapy of diverse diseases or vaccination. Mucosal delivery of nucleic acids may be particularly important for better immune protection against respiratory infections or for therapy of diseases of the respiratory tract. Mucosal delivery can be achieved in a non-invasive manner through the application of a mucoadhesive film. For optimal cargo penetration, the film could be placed to the buccal region inside the oral cavity, which consists of non-keratinized tissue. Mucoadhesive films were prepared from two layers. The active ingredient is formulated in the mucoadhesive layer made from trehalose, pullulan, and sucrose. This mixture not only exhibits excellent mucoadhesive properties but also preserves the biological activity of the incorporated agent and slowly dissolves, ensuring directed delivery to the mucosa facilitated by the second impermeable backing layer consisting of cellulose, which prolongs the film's attachment to the mucosal surface and prevents the loss of the active material to the oral cavity. This platform is compatible with various types of cargo, such as plasmid DNA, viral vectors, oligonucleotides, proteins and mRNA encapsulated in lipid nanoparticles. Cryo-EM imaging confirmed that the lipid nanoparticles containing mRNA (mRNA/LNP) remained stable and biologically active during the polymeric matrix solidification into the film and after its dissolution. To evaluate the efficacy of vaccination via the oral mucosa using mucoadhesive films, mice were immunized with mRNA/LNP vaccine formulation targeting SARS-CoV-2. The formulation elicited IgG antibody response and robust Spike protein-specific cytotoxic T-cell response. The results demonstrate that combining intramuscular prime with mucoadhesive film as booster immunization exhibits an immunological

response comparable to the standard vaccination while also inducing the secretory IgAs effective for neutralizing respiratory pathogens.

This platform provides significant benefits, such as efficient delivery, stability, and simple non-invasive administration, which could help alleviate vaccine hesitancy. Particularly for respiratory diseases, mucosal immunity could prevent early-stage infections and reduce disease transmission. Buccal delivery holds promise not only for administering modern vaccines but also for treating a wide range of diseases. Mucoadhesive films provide a new opportunity to target mucosal tissues and may be explored for therapy of pulmonary conditions such as e.g. cystic fibrosis and asthma. Furthermore, this system could be readily adapted for the delivery of various nucleic acid materials encapsulated in lipid nanoparticles for the treatment of genetic disorders and cancer.

P0451

Polypurine Reverse Hoogsten hairpins as a potential therapeutic tool for COVID-19 infection

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The RNA of SARS-CoV-2 encodes for structural proteins such as spike, membrane, envelope, or nucleocapsid, and non-structural proteins such as polyproteins pp1a and pp1b. We designed Polypurine Reverse Hoogsteen (PPRH) hairpins targeting specific SARS-CoV-2 regions for therapeutic purposes. PPRHs are non-modified ssDNA molecules made of two polypurine strands in antiparallel orientation linked by a thymidine loop and with a hairpin structure maintained by Hoogsteen bonds. PPRHs bind in a sequence specific manner by Watson-Crick bonds to their polypirimidine-rich DNA or RNA target sequences and form triplex structures that interfere with gene expression.

The effect of two PPRHs, CC1 and CC3, targeting replicase (pp1b) and spike sequences, respectively, were analyzed both *in vitro* and *in vivo*. The *Kd* values for the binding of both PPRHs to their respective targets were in the range of nanomolar. Optimal internalization (95%) in VERO-E6 cells that express the ACE receptor occurred upon transfection of 300 nM PPRH with 30µM DOTAP. In these cells, CC1 and CC3 were able to reduce SARS-CoV-2 levels by more than 90% 2-days post viral infection.

PPRHs were administered intranasally to transgenic K18-hACE2 mice expressing the human ACE2 receptor in all epithelia, using *in vivo*-JET-PEI, 24 h and 4h before and 2-, 4-, 6- and 8-days post infection with the MAD6/Wuhan strain. Mice treated with the negative control PPRH showed severe body weight loss (15%) and the highest clinical severity rating (score 75). In contrast, all mice that received CC1 survived the infection, with less than 5% body weight lost and only mild clinical signs (score 10). Additionally, CC1 administration significantly reduced the viral load in lung and brain mice tissues 4-days post infection. Altogether, our findings indicate that PPRHs offer a promising approach to improve the use of oligonucleotides for the treatment of viral diseases.

P0452

An innovative and scalable chromatographic purification approach for Modified Vaccinia Ankara (MVA) manufacturing process

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In recent years viral vectors have become widely used in the development of new vaccines and gene therapies, specifically one of the most promising and well characterized viral vector is Modified Vaccinia Virus Ankara (MVA) as promising booster in a second dose administration. It has been demonstrated that recombinant MVA is an extremely safe and efficient viral vector system. Actual strains of MVA were obtained from Vaccinia virus, members of Poxviridiae, after several passages in chicken fibroblast tissue culture in 1970s. MVA is a large complex enveloped virion containing a linear double-stranded DNA genome of 178 kbp and with a size between 220-450 nm length, 40–260 nm width and 140–260 nm thickness. Due to this very large dimension it's not possible to filter 0.22 µm the final drug substance (DS), so current cGMP manufacturing process of MVA vector need to be performed in a completely closed system with single use and sterile materials. Although most of MVA purification processes, based on clarification and tangential flow filtration steps, allow to obtain a final product in line with regulatory requirements on residuals, in terms of host-cell DNA (<10 ng/dose), one of the main bottlenecks from downstream perspectives remains the total protein amount in final product. For this reason, we developed a novel purification protocol with a chromatographic step, based on CIMmultus (CIM) uncharged columns CIM-OH (Sartorius). CIM columns are prepacked monolithic columns featuring large flow-through channels, designed for working with biomolecules, such as viruses. In particular CIM OH has a neutral ligand and allows a chromatographic separation based on hydrophobic interactions. Our purification method is based on MVA infection of avian suspension cells. After 48hpi, the virus is harvested and lysed by a single pass sonication prior to be purified from hc-DNA by endonuclease digestion. Two clarification steps were performed using a filters train with a gradient pore size to retain the main cellular debris and process related macrocomplexes. The resulting product is then loaded in a CIM OH column, after a dilution with a buffer containing $MgCl_2$, sucrose, ammonium sulfate and NaCl, in order to enable the binding of the virus with the monolithic column. A wash step is performed to enhance the contaminants clearance and finally the purified virus is eluted with a buffer without any salts. The complete purification process includes also a final step of Tangential Flow Filtration with a Hollow fiber to remove the remaining contaminants, concentrate the product to the target concentration and formulate the DS in the final buffer. The introduction of the chromatographic step allows to obtain purified product with a very low impact on step yield, reducing the total protein amount in the eluted fraction of about 8-times compared to the production of MVA without this step. Moreover, CIM OH columns are perfectly scalable for GMP production and could be used in a closed system.

P0453

Upstream process intensification for a gorilla derived adenoviral (GRAd) vector by using a perfusion system

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Recombinant adenovirus vectors are effective gene transfer tools used both in gene therapy, cancer therapy and infectious diseases. Current processes for manufacturing Adenoviral vectors (AdV) involve the use of serum free media and stirred tank bioreactors by employing HEK293 cells as packaging cell line. Cells are amplified in shake flask, wave and stirred tank bioreactors where viral production is achieved by infecting the cells with the AdV at a specific Multiplicity of Infection. Developing a process capable of producing large quantities of doses both for clinical and commercial phases has become crucial for the biotech market in recent years. Viral productivity as well as bioreactors size determine the number of doses that can be produced for each batch. Although large scale stirred tank bioreactors are available for cell cultivation and production phases, huge amount of cell biomass is needed for the inoculation, thus increasing both the timing for cell amplification and the overall manufacturing costs. One strategy to boost viral productivity is to increase the concentration of the cells at the infection phase thus improving the total volumetric productivity. However, a challenge with this strategy is the cell density effect, where higher cell densities often lead to lower per cell productivity due to lack of nutrients and toxic cell metabolites accumulation. The perfusion process, which involves continuously replacing exhausted medium with fresh medium, can be a successful solution to achieve higher cell density during the infection phase. In this study, we present an intensified upstream process based on proprietary HEK293 cell line adapted to suspension growth in a small-scale 2L Stirred Tank Bioreactor for the production of Gorilla-derived Adenoviral vector (GRAd). Through the use of a Perfusion system, we achieved a high cell density culture of approximately 1E+07 cells/mL. Furthermore, infecting the cells at a density of 2E+06 cells/mL after perfusion led to a threefold increase in the total volumetric productivity of the vector compared to standard processes, without affecting vector quality. Cell growth, viability, and metabolite profiles were monitored throughout the process to characterize cell culture, demonstrating that medium exchange can sustain exponential cell growth maintaining high cell viability. This study establishes that a successful and reproducible intensified process can be applied for the production of GRAd vector in HEK293-derived cell line. This could lead to a reduction in vaccine manufacturing time and an increase in the number of doses produced per batch at the manufacturing scale.

P0454

Enhanced thermostability of a gorilla-derived adenoviral (GRAd) vector via E4 region chimerism

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ReiThera has developed a COVID-19 vaccine based on a gorilla adenoviral vector (GRAd-COV2). The vector is replication-defective due to the deletion of the E1 region; two additional deletions have been added in the E3 and E4 regions replacing E4 with human Adenovirus 5's E4 orf6. This backbone is referred to as backbone (c). The GRAd-COV2 (c) vaccine has undergone Phase I and Phase II clinical trials. The GRAd-COV2 (c) vector was initially selected for its high productivity, crucial for rapid vaccine dose production. However, other adenoviral platforms also widely used in clinical settings retain an intact, yet chimeric, E4 region. Here we show GRAd-COV2 (b2) - that retains GRAd32's E4 orf1, orf2, and orf3, while replacing orfs 4, 6, and 6/7 with those from human Adenovirus 5 - demonstrated superior thermostability, when compared to GRAd-COV2 (c), enabling storage at 2-8°C, which is advantageous for global distribution. In vitro comparability studies assessed productivity, infectivity, and expression. GRAd-COV2 (b2) exhibited satisfactory productivity, slightly lower than GRAd-COV2 (c) but meeting the necessary criteria for large-scale manufacturing. Infectivity assessments indicated comparable performance between the two vectors, with a marginally better ratio for GRAd-COV2 (b2). Expression analysis via Western blot and FACS showed no significant difference in Spike protein expression levels between the two vectors. Preclinical studies evaluated immunogenicity in BALB/c mice, with both vectors inducing comparable Spike-binding antibody titers and T cell responses. Overall, the GRAd-COV2 (b2) vector demonstrated a good immunogenicity, productivity, and infectivity. In addition, the backbone b2 provide a more thermostable product, a property linked to the possibility of storing formulated vaccine lots at 2-8°C, which is extremely beneficial for transport and storage in different countries worldwide. These important characteristics support the use of this backbone for the Phase I clinical trial for GRAdHIVNE1.

P0455

Development of a reverse phase chromatography assays for quantification of detergent residuals in vaccine purification process

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During the purification process of viral vector vaccines, detergents such as Polysorbate 20 (PS20) and Domiphen Bromide (DB) are used to stabilise the product and facilitate the precipitation and subsequent removal of undesired molecules. The use of Domiphen Bromide as a cationic detergent in the purification steps of vaccine formulation is becoming increasingly important, particularly due to its high selectivity for host cell DNA. Polysorbate 20 is used for both its surface adsorption prevention function and its role as a stabiliser against protein aggregation. Considering the importance of these compounds, their monitoring during the purification process plays a central role quality-wise to ensure that these compounds are accurately eliminated during the relevant purification steps. Since no HPLC methods for identifying these compounds in vaccine formulations were available in the literature, our aim was to develop a method that could be useful for the constant monitoring of these residuals in the purification steps. High performance liquid chromatography (HPLC) has a dominant position among the modern techniques in both the qualitative and quantitative drugs analysis. After appropriate sample preparation it is also commonly used for the determination of drugs and metabolites in biological material. DB was isolated using an Acquity UPLC BEH C18 column (2.1mm x 50mm, 1.7um) as the stationary phase, isocratic elution and a TUV detector set to 270nm wavelength. Correlation coefficients of calibration curves pointed out that they were linear within the examined concentration range. The results show that most of the Domiphen Bromide is efficiently

eliminated during the purification steps, showing a concentration in the final product <2.5ug/mL, where 2.5ug/mL is the LOQ and LOD limit for the test, and therefore that 98% of the compound gets cleared compared to the initial quantity introduced in the process. The stationary phase chosen for the isolation of PS20 is a Kinetex C8 (100 x 2.1mm, 2.6 um) with gradient elution of water and acetonitrile. Due to the nature of PS20, a sample preparation involving carbon chain cleavage and subsequent derivatisation had to be developed for its identification. The resulting molecule can be identified using an FLR detector with an excitation wavelength of 340nm and an emission wavelength of 395nm. As with DB, the correlation coefficients of the calibration curves for PS20 showed their linearity over the concentration range studied. The results show that most of PS20 is efficiently eliminated during the purification step, resulting in a 98% reduction in the concentration of PS20 in the final purified Drug Substance (DS) compared to the initial virus harvest in process sample. These data should be taken into consideration in light of the fact that the PS20 concentration in the final DS is not detectable and that the LOQ of the method is 2.5 ug/mL while the LOD is 1.25 ug/mL.

P0456

The MHC MACSimer technology combines flexibility, quality and releasabilty for the detection of antigen specific T cells

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Antigen (Ag)-specific T cells play an essential role in the immune responses towards cancer, infectious diseases, and autoimmunity. Furthermore, the precise analysis, isolation and characterization of these cells is crucial for the development of cellular TCR T cell therapies, cancer- or virus-specific vaccinations, and patient monitoring. Given the individual component of Ag-specific T cell responses ("MHC/HLA" restriction), researchers need innovative and flexible tools providing them with high-quality results to enable effortless integration of Ag-specific T cell responses into translational workflows.

We have therefore designed new reagents, the *MHC MACSimer FleX Kits* and *peptide-loaded MACSimers*. The novel construct design of the MHC multimer product is overcoming major challenges of Ag-specific T cell analytics. They feature superior specificity and fluorescence brightness through an optimal balance of MHC molecules and fluorophores, enabling the detection of even extremely rare T cell subsets with highest reliability.

To enable the integration into translational workflows our MHC MACSimer reagents feature

1. A flexible peptide-loading technology in the case of our *MHC MACSimer FleX Kits*, allowing for extensive T cell epitope screening.
2. The REAlease® technology for our *MHC MACSimer FleX Kits* and *peptide-loaded MACSimers* to obtain label-free T cells after enrichment, e.g., for multiple sequential sorting steps or functional downstream assays.
3. A comprehensive collection of *MACSpep Single Peptides* covering a broad variety of T cell epitopes related to cancer, infection, and autoimmunity, delivering the ideal accessory to our *MHC MACSimer FleX Kits*.

We show that both, our *MHC MACSimer FleX Kits* as well as our *peptide-loaded MHC MACSimers* feature superior performance when staining and analyzing shared cancer antigens, or virus-specific T cells for both, CD4+ and CD8+ T cells of different origins (PBMC, whole blood samples, or dissociated organs) and comparing them to other state-of-the-art MHC multimer reagents. Furthermore, we demonstrate that *MHC MACSimer* reagents can be perfectly combined with antibody panels allowing for analysis of e.g., T cell exhaustion or differentiation to get the most information out of the rarest T cells. Combining them with fluorochrome-specific magnetic beads using MACS technology leads to extremely pure (>90%) and high-yield-enriched T cell populations, displaying the intended T cell phenotype. *MACSpep Single Peptides* come with a tailored MHC loading protocol and alternatively can be used for epitope-specific activation and expansion of T cells ex vivo. In conclusion, our three reagent classes enable Ag-specific T cell analysis, enumeration, and isolation and cover the need for flexibility, stability, and standardization.

P0457

Inhibition of human adenoviruses by Argonaute 2 alone and in combination with artificial microRNAs

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Human adenoviruses are double-stranded DNA viruses that can cause life-threatening infections in immunocompromised patients especially after solid organ or hematopoietic stem cell transplantations. Numbers of recipients of such transplants are constantly rising. However, the options to treat human adenovirus infections are limited and therapy sometimes lacks efficacy and is moreover associated with toxic side effects. Consequently, investigating alternative approaches for the therapy of life-threatening adenovirus infections is necessary. In this study we studied the inhibition of human adenoviruses by artificial miRNAs (amiRNAs) and how the degree of inhibition could potentially be increased. In particular, we investigated the effect of concomitant overexpression of Argonaute 2 (AGO2), a central component in the RNA interference (RNAi) pathway mediating the interaction between endogenous miRNAs or amiRNAs and their respective target mRNAs. The rationale for this approach is based on the fact that adenoviruses are known to saturate endogenous AGO2 proteins to inhibit cellular RNAi. Inhibition of the cellular RNAi machinery is thought to generate an environment that is beneficial for the virus. We demonstrated that AGO2 overexpression in infected cells alone has a significant negative effect on viral replication *in vitro*, probably by resolving the bottleneck in the RNAi pathway generated by the virus and restoring endogenous RNAi function. Furthermore, AGO2 overexpression-mediated re-establishment of functional RNAi strongly increased the function of amiRNAs designed to target the mRNA coding for the viral preterminal protein (pTP), an essential component in adenoviral DNA replication. Adenovirus vector-mediated delivery and co-expression of AGO2 and amiRNAs into infected cells heavily decreased both adenoviral genome copy numbers and numbers of infectious virus particles *in vitro*. On average, AGO2 overexpression improved the amiRNA-mediated inhibition of adenoviral replication by two orders of magnitude.

P0458

LNP-mediated CRISPR/hfCas12Max gene-editing to target covalently-closed-circular DNA for chronic hepatitis B infection

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Hepatitis B virus (HBV) infection is one of the most prevalent infectious diseases worldwide. The hepatotropic, non-cytopathic DNA virus, HBV, generates a covalently-closed-circular-DNA (cccDNA) intermediate in the nucleus of infected cells, as well as integrated sequences that act as templates for viral transcription and protein production. Although currently approved antiviral therapies with nucleoside analogs and immunomodulators inhibit replication of HBV DNA in capsids present in the cytoplasm of infected cells, they fail to reduce or eliminate nuclear cccDNA as well as cure chronic hepatitis B. Gene editing holds the great potential to target all forms of viral DNA, including cccDNA, and may offer a new treatment option for functional cure or even better clinical outcomes. Several CRISPR/Cas DNA editing-based therapeutics are currently under preclinical development to achieve this therapeutic goal. Building on a proprietary AI-based HG-PRECISE® platform for novel CRISPR nucleases, we have developed high-fidelity Cas12Max (hfCas12Max), an engineered Cas12i variant with high activity and specificity, delivered by lipid nanoparticle (LNP) which targets the HBV genome. Effective guide RNAs (gRNAs) were initially selected and screened within the PiggyBac(PB)-HBV-HEK293 system. A functional gRNAs screen was performed in HBV-integrated cell lines and primary human hepatocytes (PHHs) infected with HBV. To evaluate the *in vivo* efficacy of the hfCas12Max nuclease, we developed an episomal adeno-associated virus (AAV) mouse model harboring a 1.3-fold HBV genome, which serves as a surrogate for cccDNA. Clinically relevant delivery was achieved through the systemic administration of LNPs encapsulating hfCas12Max mRNA and gRNAs. Our study identified leading gRNA candidates efficiently targeting the hepatitis B surface antigen (HBsAg) and associated genes within HBV-integrated hepatic cell lines. Upon delivery via the LNPs, the hfCas12Max targeting significantly decreased cccDNA, HBV-DNA, and HBsAg levels in HBV-infected primary human hepatocytes. In the AAV-HBV mouse model, we observed sustained and substantial reductions in HBsAg expression (up to 2.5 log) and serum HBV DNA (~3log) following systemic delivery of varying doses of the CRISPR/hfCas12Max system mediated by LNP, suggesting that the CRISPR/hfCas12Max system could disrupt the HBV-expressing templates *in vitro* and *in vivo*. Our results laid the foundation for a potential CRISPR/Cas DNA editing-based therapy and warrant further development of a functional curative CRISPR/hfCas12Max gene-editing therapy for treating chronic hepatitis B infection.

P0459

Genetically encoded sensor circuits for the development of therapies for infectious diseases: a theragnostic approach

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Proteases play essential roles in cell survival and death. Shifts in proteolytic systems underlie multiple pathological conditions such as infectious diseases, cancer, or neurodegenerative disorders. As such, proteases are a major focus of attention as potential drug targets or as diagnostic and prognostic biomarkers. Synthetic circuits based on proteolysis can regulate therapeutic actions in gene and cell therapies for the treatment of both acquired and hereditary diseases.

This work explores proteolytic biosensors to develop treatments for infectious diseases. These biosensors can be used as tools for antiviral drug discovery or, as theragnostic circuits to deliver controlled and effective gene therapies.

Many viruses encode proteases, thus our first biosensors were developed targeting infectious diseases. We have successfully implemented two types of cell-based biosensors. The first generation consisted of a single genetic module providing sensor and output signal. Our second novel generation of biosensors was developed with an extra module of control, where the viral protease activates a protease-conditional recombinase (ProRec) sensing molecule that activates the transcription in a second output module. GFP was used as proof-of-concept output signal in both cases. Both generations of sensing modules were conditionally inactivated through the use of protein distortion.

A whole-cell biosensor for the detection and quantification of infectious human adenovirus (HAdv) was successfully developed, with fluorescence emission (re)activation upon infection, allowing detection and quantification of infectious virus. The sensitivity and dynamic range were, however, limited. The limit of detection (LOD) was a multiplicity of infection (MOI) of 0.16, corresponding to 10^5 I.P. mL^{-1} in our experimental conditions. The later LOD is similar to flow cytometry-based protocols that make use of labeled HAdv.

The second-generation ProRec biosensors showed low background and high output signal. It demonstrated robust detection of rare events (high sensitivity). Circuits for the successful detection of several viruses were developed, namely tobacco etch virus (TEV), HAdv, and rhinovirus (HRV). Whole-cell biosensor biosensors circuits showed full sensor activation for TEV and HRV (high GFP intensity).

The ProRec circuits provide versatile and regulatable output signals, enabling not only detection (biosensing) but also the development of targeted advanced therapeutics (theragnostic). Currently a ProRec is being coupled with antiviral therapeutic modules to provide advanced gene therapy theragnostic treatments. Future works aims at the development of circuits for the treatment of other acquired and inherited diseases.

P0460

In Vivo Production of Anti-HIV Antibody from Engineered Primate Hematopoietic Stem and Progenitor Cells

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Immunization approaches against HIV are currently aimed at durable production of broadly neutralizing antibodies (bnAb). Both passive transfer and non-integrating virus-mediated delivery of anti-HIV bnAb transgenes have demonstrated protective properties in non-human primate (NHP) models of infection, but these approaches lack capacity for long-term protection and confer host anti-viral and anti-bnAb immune responses. To address these issues, we engineered NHP hematopoietic stem and progenitor cells (HSPC) by targeting the immunoglobulin heavy chain locus, *IGH*, using Cas12a ribonucleoprotein complex to integrate a non-viral transgene for endogenous expression of the bnAb 10-1074. We transplanted these non-virally engineered NHP HSPC into sub-lethally irradiated, neonatal immunodeficient mice, referred to here as primatized mice. Using this model, we previously demonstrated that engineered HSPC are capable of engrafting and developing *in vivo*. Edited cells can be found in circulation for up to 20 weeks, although the fraction of cells with large insertions at the target locus decline over time (n=8 mice). We hypothesize that NHP-derived cells expressing a reporter transgene undergo little to no clonal expansion, and that the expression of an antibody transgene would require lymphocyte differentiation and immunization. In this study, we report that the anti-HIV bnAb 10-1074 can be expressed *in vivo* over 18 weeks following transplantation and antigen boosting using gp120 antigen in primatized mice. Detectable plasma anti-HIV Ab titers of up to 2.38 µg/mL were observed in 1 out of 5 mice that received doses of 5.0×10^5 NHP-CD34$^+$ cells at the time of transplant. This Ab-producing animal displayed the cohort's highest engraftment levels with up to 20.6% NHP-CD45$^+$ cells in its peripheral blood. It also displayed the earliest rise in T cell populations with NHP-CD3$^+$ cells accounting for 34.5% of the NHP lineage as early as 10 weeks and 77.0% at week 16. Surface expression of the bnAb transgene linker was detected in all primatized mice, albeit at low levels <1.16% of NHP-derived cells in the peripheral blood. The mouse with detectable titers did not display significantly more bnAb-producing cells in peripheral blood, suggesting the majority of these cells are not in circulation. During necropsy, multiple tissues were assessed, revealing thymus development with high NHP cell content (mean=95.7%) in 80% of primatized mice, as well as potential plasmablast or plasma cell presence in one animal with 2.76% NHP-CD138$^+$ cells in the bone marrow. Sequencing of these tissues is underway to reveal the localization of edited cells. Importantly, these findings suggest that *in vivo* production of engineered antibodies can be achieved by targeting HSPC without the use of viral vectors. Future studies will assess whether transplanting higher numbers of engineered HSPC will result in a larger fraction of mice expressing bnAb. These results will guide *in vivo* engineering approaches and help predict the target editing levels necessary for clinically relevant outcomes.

P0461

Improved viral vector vaccine stability by mitigating interaction of viral particles with glass surfaces

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Long term vaccine stability at refrigerated temperatures (2-8°C and ambient) is critical to ensure a global reach of the vaccine using a standardized supply chain. During Ad26 clinical vaccine development, some instability at 2-8°C was observed which triggered a root cause investigation. There was a risk that the target shelf life of at least 2 years at 2-8°C would not be met.

The root cause was identified as adsorption of viral particles (vp's) to the glass surface. This phenomenon is in particular observed upon inversion of the vial at 2-8°C due to the additional loss of vp's to unexposed glass surface. Various innovative analytical tools were applied including spectroscopic kinetic monitoring of the loss of the vp's directly inside the 2R glass vials. This was enabled by creating custom 3D printed accessories to fit the equipment. Models were created that allowed further predictions on the amount of vp's adsorbed, dependent on volume, titer and vial type. Additional orthogonal analytical methods were applied to confirm the results, such as visualizing adsorbed material by dyes (staining) on the glass walls as well as determining the vp content using VPqPCR and CZE.

A dual mitigation was implemented for the Ad26-based vaccines to avoid further loss of vp's during shelf life of the vaccines to the glass surface and to enable a sufficient long shelf life at refrigerated conditions.

i) For **single dose vaccines**, the primary container was changed to siliconized glass vials.

Large scale manufactured drug product vaccines showed an improved stability profile at 2-8°C and confirmed that the proposed mitigations were successful. The stability is now in line with reaching a target shelf life of at least 24 months.

ii) For **multi dose vaccines**, an overage was applied to compensate for the process loss in combination with maximizing the fill volume for the 2R glass vial. As such, upon inversion, any additional loss of vp's to glass surface was negligible as the vials were filled to the max, allowing administration of 5 doses of 0.5mL from every vial. This was critical as the standard type 1 glass vials were available in large amounts during the onset of the covid pandemic.

The above findings can also be applied to cell and gene therapy products.

P0462

Immune response evaluation of pyro-drive jet injector-delivered nucleic acid-based vaccines

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A steady development of drug delivery system is indispensable not only in gene therapy but also in extensive medical technology. A pyro-drive jet injector (PJI) is a novel injection device which shoots out a solution by pressure of gunpowder explosion. PJI is able to effectively deliver messenger RNA and plasmid DNA to dermal tissue in animal models. In recent years, mRNA vaccines were successfully developed and approved for emergency use to fight coronavirus disease. However, the effect of DNA vaccines against SARS-CoV-2 is considerably lower than that of mRNA vaccines. In this study, the immunogenic potential of pDNA and mRNA vaccines were evaluated in mice model using PJI. PJI was used to deliver naked mRNA and pDNA and their efficacy in inducing antigen expression and immune responses was assessed. Our results showed that PJI efficiently delivered mRNA into the skin, and a smaller effective dose than that of pDNA injection was required to achieve similar levels of antigen expression. The PJI-delivered CpG-free pDNA vaccine efficiently induced antigen-specific antibody production and a cell-mediated IFN-γ response compared to the mRNA vaccine, as well as the upregulation of inflammatory cytokines (IL-6, IFN-γ, and IL-1β) in the skin and lymph nodes. However, the intradermal mRNA vaccine was significantly less immunogenic than the standard intramuscular mRNA-lipid nanoparticle vaccine, despite equivalent mRNA dosages. In conclusion, PJI-delivered DNA vaccine strongly elicited antigen-specific antibodies, cell-mediated immune responses, and pro-inflammatory cytokines, and further research is needed to refine and elucidate the effects of PJI on both mRNA and DNA vaccinations.

P0463

Development of novel genetic vaccine platforms: from the idea to GMP production

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The COVID-19 pandemic has demonstrated the urgent need to develop versatile vaccination platforms that could be quickly implemented for infectious diseases and to respond promptly to future pandemic outbreaks. It is of utmost importance to provide a vaccine in a time-critical manner for such diseases, as the frequency of such epidemics and pandemics as we see them today, will be heavily increasing due to rise in global travel, global warming, increase in population density, penetration into previously uninhabited areas and animal trade. Nucleic acid vaccines, such as those based on mRNA are endowed of these features.

To address the urgent need to find solutions to the SARS-CoV-2 Pandemic, Takis has developed COVID-eVax, a vaccine approach based on genetic engineering and DNA electroporation as part of the X-eVax platform, previously developed. The project started in 2020 and consisted of the molecular design of the vaccine, the development of the reagents and tests necessary to test its effectiveness and the experiments in animal models. Subsequently, GMP-grade material (Good Manufacturing Practices) was produced, all regulatory studies were conducted (toxicology, biodistribution, immune response) and finally a phase 1 study in humans, which ended in December 2021, achieving all the objectives set and providing the basis for evaluations in other applications.

DNA vaccines advantages are: (1) simple and quick production of DNA encoding the antigens by PCR or synthetic methods (potential game-changers for new variants especially vaccine resistant strains), (2) easy large-scale production, (3) safety compared to inactivated virus vaccines, and (4) higher thermostability (minimal cold-chain requirements), which is an issue with some vaccines. The DNA-based platforms offer great flexibility in manipulating the encoded vaccine antigen and have a great potential for accelerated development. Recently, the first DNA vaccine against SARS-CoV-2 (ZyCov-D) has been registered in India for human use; moreover, DNA vaccines have been extensively tested in multiple clinical trials in the oncology field and are commonly used in veterinary medicine. These vaccines (as opposed to mRNA-based vaccines) are stable, do not require cold-chain supply, and can easily be produced in large amounts in bacteria. All these advantages make this platform technology an attractive tool, as it overcomes several shortcomings of alternative approaches (e.g., complex production processes, stability issues, purchase price). Besides the classical plasmid form of DNA, we have also developed a linear form of DNA, encoding only for the antigen of interest. This novel form of nucleic-acid vaccine has already proven to be efficacious in preventing SARS-CoV-2 infection in feline and ferret animal model. In this presentation, opportunities and challenges of DNA-based vaccines and Takis biotech experience will be discussed.

P0464

Development of an Adenovirus-Based Vaccine Vector Encoding the SARS-CoV-2 Spike Protein against COVID-19

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The urgent need for a safe and effective vaccine against COVID-19, caused by the SARS-CoV-2 virus, has driven the exploration of various vaccine platforms. Among these, viral vector-based vaccines, particularly those utilizing adenovirus vector, offer significant advantages. Adenovirus-based vectors are characterized by their broad tissue tropism, well-understood vector genome, high transgene capacity, natural adjuvant effect, and ability to induce robust transgene-specific T cell and B cell responses. This study focuses on the development of a human adenoviral vector based on Serotype 5 encoding the SARS-CoV-2 Spike protein as potential vaccine candidates. Using Gateway® Cloning Technology, we constructed a human adenoviral vector encoding the SARS-CoV-2 Spike protein (Ad5Spike). The Ad5Spike vaccine vector was generated by transient transfection of the pAd5Spike expression plasmid into 293A cells via the calcium phosphate-mediated method. Post-transfection, the Ad5Spike virus was purified and concentrated through cesium chloride density gradient ultracentrifugation. For immunization, the vector was intraperitoneally injected at different concentrations (10^8, 10^{10}, and 10^{12} viral particles) into 6-8-week-old female BALB/c mice in 10 mM Tris-Cl buffer. Both cellular and humoral immune responses were evaluated at 30- and 90-days post-immunization using ELISA, ELISpot, and neutralization assays. To assess the humoral immune response IgG ELISA was performed and these mice exhibited producing of serum anti-spike IgG. Furthermore, IL-2, TNF-α, and IFN-γ sytocines were significantly higher compared to the control groups. Espesically cytotoxic T cell responses (IL-2 and IFN-γ) were detected even at 90 days post-vaccination. Finally, neutralization tests conducted using 3rd generation lentiviral-based pseudovirus demonstrated that the vaccinated mice, particularly those in the high dose group, were effectively protected against potential viral infection and exhibited strong neutralizing activity against SARS-CoV-2 in splenocytes. In conclusion, the development of the Ad5Spike vectors shows great

promise as a vaccine candidate against COVID-19. The ability of this vaccine to induce both humoral and cellular immunity suggests its potential to provide protective immunity for at least 90 days against SARS-CoV-2 (This study is supported by grants from Akdeniz University Scientific Administration Division, Grant No. TDK-2022-6067).

P0465

Programmable RNA nuclease mediated destruction of SARS-CoV-2 RNA to prevent replication and spread ("PRiMeD COVID")

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The SARS-CoV-2 coronavirus emerged in 2019 causing a global pandemic of severe respiratory complications. Although vaccination improves immunity against virus infection, the evolution of new SARS-CoV-2 variants remains a major limitation for controlling the virus spread. To reduce the risk of zoonotic related or un-related virus outbreaks in the future, a pan-coronavirus antiviral approach is being developed to combat not only SARS-CoV-2 but also various other coronaviruses. Our project aims at inhibiting intracellular viral replication by using the recently developed CRISPR-Cas13d or CasRx system. We have designed CRISPR guide RNAs (crRNAs) targeting the conserved replication-related regions in the genome of different coronaviruses. The target regions include the RNA-dependent RNA polymerase (RdRp), the 5' and the 3' untranslated regions (5'UTR and 3'UTR). For the screening of crRNAs, we constructed a stable veroB4 reporter cell line expressing a destabilized GFP on the same transcript as SARS-CoV-2 sequences of RdRp, 5' and 3' UTR. Thereby, specific degradation of the viral mRNA transcript will result in a reduction of GFP expression. We have confirmed the effect of CasRx/crRNAs on SARS-CoV-2 restriction using this reporter cell line, showing 37%, 76% or 82% decrease in GFP intensity in cells treated with CasRx mRNA and RdRp, 5'UTR or 3'UTR-targeting crRNAs, respectively. To demonstrate efficient reduction of viral load in SARS-CoV-2 virus, we have transfected our most efficient crRNAs in a veroB4 cell line stably expressing CasRx, then infected cells with wildtype SARS-CoV-2 virus. 24 hours post-infection, we observed up to 60%, 60% or 50% reduction in viral titer by knocking down RdRp, 5'UTR or 3'UTR, respectively. Interestingly, these results suggest that, in contrast to our results using the reporter cell line, CasRx/RdRp-crRNA is the most promising approach to inhibit SARS-CoV-2 replication. To enable *in vivo* lung delivery of the optimized CasRx/crRNA payload, we are investigating Adeno-Associated Virus serotype 6 (AAV6) and Lipid Nanoparticles (LNP) as delivery vectors. AAV6 triple-mutant vector (AAV6.2FF) expressing a firefly luciferase gene (FLuc) was applied to BALB/c wt mice via intratracheal or intranasal instillations. AAV6.2FF vector showed a robust FLuc expression exclusively in the thorax (lung) region up to 12 weeks post-administration with minimal immune response in the lungs. Since short-term expression of our antiviral treatment might be more desirable in most clinical settings, we are currently developing a novel LNP formulation allowing for topical aerosol delivery of CasRx/crRNA to the lungs. We then plan to intratracheally administer LNP encapsulated Fluc-P2A-GFP mRNA into mice to validate LNP vector functionality and cellular localization of expression in the lung. In the future, the most effective inhalation based-delivery will ultimately be employed to deliver CasRx/crRNA payload to validate its antiviral activity prior to and post coronavirus infections in the mouse model.

P0466

Fluorescent NanoSight NTA for Viral Vector Analysis: From Titer Reporting to Nucleic Acid Detection

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Nanoparticle tracking analysis (NTA) has emerged as a novel technique generating high-resolution size and concentration data of various biologics, synthetic particles and viral vectors. A quick and effective titer determination is crucial during troubleshooting or formulation optimization. The newest **NanoSight Pro** helps to delve deep into detailed characterization of gene therapy carrier as a comprehensive and often alternative method to bioassays, providing the essential size and titer information in minutes, with visual validation of sample heterogeneity. With superior sensitivity and its fluorescent detection capabilities it is possible to validate and identify vectors and their subpopulations quickly and efficiently.

This study shows how NanoSight Pro is used in general characterization of vial vectors and demonstrates the potential of the NanoSight Pro specialized fluorescence mode for in depth understanding of lentivirus and MVI characteristics. The general membrane and more specific antibody and nucleic acid tagging were tested.

The NanoSight Pro successfully detected each of the three different labelling strategies: Firstly, it successfully detected membrane labelling in order to recognize vectors. Secondly, the presence of VSV-G was measured for decoding vector's identity. Finally, the NanoSight Pro revealed a signal of nucleic acid for discovering vector's secrets.

The results demonstrate how NanoSight Pro, and its specialized fluorescence mode provides detailed information in viral vector characterization. It reveals size, titer and labelling efficiency for various applications including the detection of vector membrane, specific markers as well as cargo for a better understanding of viral vectors characteristics and providing greater inside in the optimization processes.

P0467

High efficiency of CAR-transduction utilizing Hillgene's innovative lentivirus vector platform

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Introduction

Lentiviral vectors (LVV) are often used as target gene delivery vectors in immune cell therapy due to their packaging capacity, low cytotoxicity and immunogenicity, wide infection spectrum, and stable expression. The increasing diversity and complexity in the needs for therapeutic genes editing poses the technical challenges in the viral vector design, process development, and large-scale production, resulting in long R&D time and high cost of viral vector production.

Purpose and significance

The current market has limited lentivirus products that cannot meet the scientific research needs of rapidly developing cell therapies. Based on the in-house viral vector platform, Hillgene has established lentivirus production processes and product quality standards for CAR-T, NK, and TIL cells. The off-shelf lentivirus products for different targets cell therapies are available on the market to support cell therapy R&D.

Key technologies and methods

(1) The Hillgene's HiLenti four-plasmid lentiviral packaging system has completed the establishment and verification of the three-tiered bacterial strain banking for three packaging plasmids (pHi001/pHi002/pHi003). The results of characterization and testing of banked cells are compliant with regulatory requirements. The yield and quality of products utilizing the HiLenti plasmid system meet the needs of commercialization.

(2) Hillgene has established a scalable HiLenti serum-free suspension 293T platform to manufacture the lentiviral vector consistently. Three-tiered banking of 293T cell line can decrease the variation of titer under the same manufacturing process. The results of characterization and testing of banked cells are compliant with regulatory requirements.

(3) Hillgene has developed a more effectively transduction platform utilizing BaEV envelope protein in lentiviral vector packaging system, comparing to the conventional VSV-G envelope protein, providing a unique solution to ex vivo gene-editing cell therapu development. The lentivirus packaged by this envelope platform be used to improve the purity and titer of the lentiviral vectors.

(4) Optimization of lentiviral processes and key process parameters, technical challenges such as low yield, low purification process yield, and difficulty in removing impurities have been greatly improved, achieving the premium quality of lentivirus products with high yield and titer, high recovery rate, and low impurity level.

Conclusion

The off-shelf lentivirus for CD19 CAR-T, EGFR TIL, CD19CAR-NK, and BCMA & IL15 based CAR-NK products produced by Hillgene are now available on the market, providing global cell therapy customers with a full range of off-shelf and customized lentiviral vector services.

P0468

Prolonged activity of the transposase helper may raise safety concerns during DNA transposon-based gene therapy

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Long-term gene expression and clinical benefits often require the integration of therapeutic genes into the chromosomal DNA of target cells during gene therapy interventions. From the early stages of gene therapy, viral vectors have represented the majority of vehicles for transferring genes to human cells. However, during the first decade of the 2000s, insertional oncogenesis emerged as a major limitation in retrovirus-based gene therapy protocols treating primary

immunodeficiencies. It became clear that, biased genomic vector integration patterns and the potential of the vector to transcriptionally deregulate nearby genes, collectively increase the likelihood of insertional oncogenesis. DNA transposon-based gene delivery vectors represent a promising new branch of randomly integrating vector development for gene therapy. Among their other potential benefits, their use is also expected to reduce the risk of insertional oncogenesis. To investigate this further, we performed gene therapy interventions with transposons in a preclinical mouse model.

For the side-by-side evaluation of the *piggyBac* and *Sleeping Beauty* systems—the only DNA transposons currently employed in clinical trials—during therapeutic intervention, we treated the mouse model of tyrosinemia type I with liver-targeted gene delivery using both vectors. For genome-wide mapping of transposon insertion sites we developed a new next-generation sequencing procedure called streptavidin-based enrichment sequencing, which allowed us to identify approximately one million integration sites for both systems. We revealed that a high proportion of *piggyBac* integrations are clustered in hot regions and found that they are frequently recurring at the same genomic positions among treated animals, indicating that the genome-wide distribution of *Sleeping Beauty*-generated integrations is closer to random. Besides, we confirmed that, although *piggyBac* integrations are biased toward genes, most of the gene body is less attractive to them, while the 5′ regulatory regions are strongly favored. Comparing the numbers of supporting next-generation sequencing reads of vector integrations in short- and long-term monitored groups of treated animals we found no signs of insertional oncogenesis using either transposon system. Importantly, by investigating the distribution of vector integration sites with bioinformatics tools, we revealed that the *piggyBac* transposase protein may exhibit prolonged gene transfer activity, and proved this notion using time-shifted delivery experiments of the transposase and transposon components. Such prolonged activity of the transposase helper predicts the risk of oncogenesis by generating chromosomal double-strand breaks and other rearrangements.

This work offers a potential mechanism to explain the adverse events seen in the clinical trial of first-in-human administration of *piggyBac*-modified chimeric antigen receptor (CAR) T cells. During this clinical trial, the formation of product-derived CAR T-cell lymphomas was reported, but insertional oncogenesis was not confirmed as the mode of tumor induction. The continued gene transfer activity we revealed here explains both the observed genetic changes and tumor induction during the first clinical trial of *piggyBac* -based CAR T cell administration. Such safety concerns associated with prolonged transpositional activity draw attention to the importance of squeezing the active state of the transposase enzymes into a narrower time window.

P0469

PacBio long-read sequence analysis of HIV-1 based lentiviral vector particles identifies packaging of multiple aberrant genomic transcripts

Lentivirus vectors (LV) offer permanent delivery of therapeutic genes to the host through an RNA intermediate genome. They are one of the most commonly used vectors for clinical gene therapy of inherited disorders such as immune deficiencies. One of the most difficult challenges facing their widespread use to patients is the large-scale production of highly pure vector stocks. To improve vector production and downstream purification there has been recent investment in the UK and worldwide to establish GMP licenced centres for manufacture and quality control.

Concerns regarding these vectors include their target cell specificity and tropism, how to regulate gene expression of the therapeutic payload and their potential side effects. Surprisingly, little is known about the full nucleic acid content of LV despite being used in several clinical trials. With the potential for side effects in mind, it is important to identify the exact contents packaged within these particles. In this study, we used highly sensitive PacBio long distance, next generation sequencing of reverse transcribed vector component RNA to investigate in detail recombinant HIV-1 particle components generated by human 293T packaging cells. We describe findings of nucleic acids other than the recombinant vector genome, including human endogenous retroviral sequences known to be involved in pathogenesis, that may be delivered during gene transfer and suggest removal of these unwanted components should be considered before clinical LV application.

P0470

Development of a highly reliable and specific assay for determining infectious titre of lentiviral vectors

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Along with the wide use of lentiviral vectors (LVVs) in cell and gene therapies, there comes the increasing need for accurate and robust analytical methods to validate the potency and quality of the vectors produced. The infectious titre assay, a critical assay for evaluation of LVVs, is inherently variable across experiments and different labs, primarily because it is a cell-based assay and can be impacted by impurities in the sample matrix. At WuXi Advanced Therapies (WuXi ATU), we developed a LVV infectious titre assay based on qPCR analysis of integrated proviral copy number in the HT1080 cell line post LVV transduction. Cells are transduced in 6-well plates to ensure accuracy and a subculture step post transduction is included to eliminate the potential impact from residual plasmid DNA. To monitor assay performance and set assay acceptance criteria we use validated lentiviral reference materials for which we have generated extensive trending datasets. A common reference sample is utilised across several analytical departments at different WuXi ATU sites to ensure data comparability. In a global comparability study, we have achieved 30% inter-assay coefficient of variation (CV) across three participating sites. Despite the reliability and consistency of the assay results observed, we aimed to further improve the assay to enhance reproducibility and specificity, but also to increase throughput and allow shortening of assay timelines. By utilising ddPCR technology instead of qPCR for assay readouts, we rely on direct quantification of genome copies, eliminating the need for standard curves and significantly improving assay reliability. This is highlighted by our assay trending data over one year with inter-assay CV reducing from 45% to 25%. To eliminate overestimation of infectious titres produced by PCR amplification of residual transfer plasmid, we designed primers and probes targeting the Delta-U3 region of LVV LTR, resulting in selective amplification of vector copies that have fully integrated into the host genome. This eliminated the need for a subculture step post infection, and therefore reduced the length of the assay significantly. We optimised the DNA extraction method as it was the most labour-intensive step in the assay. By utilising QuickExtract™ DNA Extraction Solution to replace column-based extraction methods, we have increased the throughput of DNA extraction without impacting the resulting titres. Additionally, we investigated the influence of lentiviral sample volume, vessel surface area, and the number of cells during transduction, on the output measure of infectious titre. Based on the understanding of this relationship, we have established a high-throughput 48-well plate protocol which generates almost identical titres to our existing 6-well plate protocol, with an inter-assay CV of 45%. Overall, our optimisations significantly improve the robustness, specificity, and efficiency of our

LVV infectious titre assay, resulting in more effective support for cell and gene therapy applications.

P0471

Easy and Efficient Lentiviral Transduction with a Macroporous Transduction Sponge

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Engineered cellular therapies using lentiviral vectors are transforming treatments for diseases resistant to traditional methods. To enhance lentiviral transductions and reduce inefficiencies, various strategies have been utilized, such as spinoculation, polybrene, RetroNectin®, rapamycin, and prostaglandin E2. Despite their effectiveness, many enhancers impact cell behavior. Traditional cell culture vessels pose challenges as the virus must travel significant distances to reach cells, leading to viral waste and degradation.

Microfluidic systems improve biotransport by confining cells and virus to small areas. However, previous microfluidic applications required specialized chips and hardware. To overcome these limitations, we developed the Lenti-X Transduction Sponge, a macroporous 3D alginate sponge that eliminates the need for additional hardware. This sponge is made from calcium-crosslinked alginate, a GMP-compliant, FDA-approved biomaterial known for its biocompatibility and low toxicity. The gentle cryogelation process creates a sponge with uniform pore sizes ranging from 20–300 µm.

The workflow is simple: cells and virus are applied to the sponge for absorption, followed by a 1-hour incubation, media addition, and harvesting 24 hours later. Transduced cells are released by adding a chelating buffer that depolymerizes the sponge, ensuring efficient cell release. Sponge-based transductions are flexible, accommodating 1 x 10^5 to 1 x 10^7 cells, transduction volumes of 50–150 µl, and incubation times of 4–16 hours. This approach maximizes transduction efficiency across various cell types, including suspension and adherent cells.

The alginate sponge demonstrated efficient transduction of over 15 cell targets, including human primary T cells, CD34+ HSCs, NK cells, and adherent cell lines, all with high viability. Results were consistent, with a coefficient of variation less than 15%. The innovative ex vivo Lenti-X Transduction Sponge is user-friendly, scalable, and compatible with diverse cell types, providing efficiencies equal to or surpassing conventional protocols.

P0472

INSPIIRED insights: Refining our understanding of Lentiviral Vector (LV) integration and genotoxicity

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Insertion site monitoring of gene therapy clinical trials is critical for intermediate- and long-term patient safety. Changes in the insertion site repertoire of corrected cells over time can reveal an increasing clonal dominance or, in a worst-case scenario, an emerging malignancy. Platforms like the INSPIIRED (integration site pipeline for paired-end reads) pipeline can identify the exact chromosomal position of each vector integration and quantify their contribution based on the sonic-abundance method. Using this technology, we have been monitoring LV-based gene therapy trials for Fanconi Anemia (FA), Pyruvate Kinase Deficiency (PKD), and Leukocyte Adhesion Deficiency-I (LAD-I). In addition, we used INSPIIRED to obtain biosafety insights on various retroviral vector constructs tested in our *In Vitro* Immortalization (IVIM) assay for genotoxicity assessment. The joint analysis of these data combined with theoretical mathematical modeling, revealed three major findings. First, an accumulation of integrations around the transcription start site (TSS) is expected in a polyclonal random distribution. This is due to the abundance and clustering of transcription start sites across the genome. This challenges the assumption that an accumulation of insertions in proximity to TSS are associated with a higher mutagenic risk. Second, we show that shared unique insertions between patients are statistically expected, considering every patient harbors several thousands of integrations. Hence, discerning true insertions from cross-contaminations becomes complex. Third, in diseases such as FA, in which LV-modified hematopoietic cells are administered without antecedent conditioning and in which peripheral blood (PB) vector copy numbers (VCN) are lower than in disorders in which myeloablative conditioning is employed, there is a higher likelihood that some of the more limited number of modified hematopoietic cell clones could become transiently dominant. Due to the lower PB VCN, these cells account for a smaller fraction of all hematopoietic cells. Using the same thresholds for dominance as for other diseases like PKD or LAD-I, in which more polyclonal reconstitution is expected (and has been observed), would be too stringent. By addressing these critical points, we aim to refine the current understanding of insertion site data and contribute to a better consensus on lentiviral vector safety.

P0473

Expediate high-titer manufacture of recombinant Lentivirus using a platform approach

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Manufacture of high-titer and high-quality recombinant lentivirus vector is a requirement for many ex-vivo therapies (e.g. CAR-T). Developers have several considerations for selecting a manufacturing solution for the viral vector. A primary consideration is the harvest titer of the Lentivirus Upstream Process. The manufacturing process must deliver sufficient Lentivirus vector to fill the appropriate number of dose vials as well as support the regulatory testing. To meet this demand, target harvest titers between 10^7 and 10^8 transducing units per ml (TU/ml) are required. A second consideration is cycle time to the first GMP batch. At the manufacturing stage, developers cannot perform extensive process optimization to reach the target harvest titer. Rather the timeline for Process Development, validation, and completion of the first GMP batch is expected to be less than 1 year. Third, developers want to be assured that their manufacturing process is compliant with regulatory guidelines. Manufacturing solutions that have addressed the regulatory needs of raw materials and process parameters are preferred to prevent repeating the process. To address these considerations, we developed an end-to-end solution for

Lentivirus manufacturing. The solution contains a transfer plasmid for cloning developer-specific genes, as well as three GMP quality helper plasmids that encode the VSV-G gene, the gag-pol genes and the rev gene respectively. The manufacturing solution also contains a high-performing GMP-banked HEK-293T host cell as well as a chemically defined cell culture media formulation for cell passage and production. Using a multivariate approach, we have identified transfection and process parameters to achieve titers as high as mid 10^8 TU/ml in small scale shake flasks and 3L bioreactors. Further, these defined processes were scalable to pilot scale systems and maintained high harvest titer. An industry standard for the Downstream processing of recombinant lentivirus is becoming established. Guidance from the industry includes clarification to remove cell debris, purification using anion exchange chromatography, and tangential flow filtration for buffer exchange and concentration. Using these guidelines, we developed a complete downstream solution for purification of recombinant lentivirus and have achieved product yields that are acceptable for CAR-T transduction. Moreover, the recombinant lentivirus produced is compliant with regulatory guidelines. This work will show how the platform approach addresses the key considerations of harvest titer, speed, and regulatory compliance during the journey of lentivirus vectors from gene to formulation.

P0474

2G-UNic genetic enhancers applied in the cellular and gene therapy field for increasing viral titres and payload expression

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2G UNic genetic elements are routinely used in plasmid vectors to obtain high-level protein expression in stably transfected CHO cells. This study uses modified versions of these elements aiming to increase Lentivirus titres and AAV payload expression.

The 2G UNic elements were introduced in multiple vectors of a widely used 3rd Generation LV platform and used to co-transfect HEK-293 derived suspension cells. LV production of the packaging cells was measured by different analytical methods. In a second project, 2G UNic elements were introduced in the transfer vector of an AAV system used to transfect HEK-293 packaging cells. Isolated AAV particles were used to transduce HEK cells and expression of the payload gene was measured.

Introduction of the 2G UNic elements in single LV vectors of the co-transfected set resulted in significantly increased LV titers. Combinations of vectors with 2G UNic elements further increased the LV titers. A specialized 2G UNic element was applied in the AAV transfer vector and AAV particles were harvested. The enhancer element significantly increased payload expression in transduced HEK cells with a constitutive CMV promoter as well as a tissue-specific promoter. The data show that 2G UNic enhancer elements could be widely applied in the cell and gene-therapy field for improving viral titres and payload expression.

P0475

Comprehensive biodistribution of Nipah virus pseudotyped lentiviral vectors in healthy mice

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Lentiviral vectors (LVs) are beneficial in gene therapy because they promote long-term expression, have broad tropism, can accommodate large transgenes, and are mildly immunogenic. Generally, LVs are pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) and allow wide cellular entry through low-density lipoprotein receptor (LDLR). However, the broad tropism of VSV-G LVs limits their suitability for targeted in vivo gene therapy. To overcome this, LVs can be pseudotyped with proteins from other viruses, such as Nipah virus (NiV), belonging to the family of paramyxoviruses. NiV tropism is achieved by pseudotyping with the truncated NiV glyco- (G) and fusion (F) proteins. Ephrin B2, the cellular entry receptor for NiVs, is highly expressed in organs like the heart and testes, thus creating an avenue for achieving tissue-specific targeting. A previous study showed that NiV-LVs bypass the liver sink, unlike VSVG-LVs when the viral vector was administered intravenously through the tail vein injection.

Our study focuses on characterising the biodistribution of NiV-LVs in healthy mice. We delivered a lentiviral vector using intracardiac injection to the mice and detected the Nanoluciferase transgene using in vivo and ex vivo bioluminescence imaging. The imaging results showed vast differences in the transduction of organs between VSVG- and NiV-LVs. Post-sacrification, the tissues were collected to further study and compare the transduction differences using vector copy number quantification and gene expression analysis. Overall, our study compares the biodistribution profiles of these two vectors, comparing their potential utility for gene therapy applications requiring tissue-specific transduction.

P0476

In-bioreactor nuclease treatment during lentiviral vector production

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Nuclease treatments are routinely applied to viral vector batches to minimise the amount of contaminating plasmid and host cell DNA in the final product. These are usually applied post-harvest, but pre-purification, in a separate 37°C incubation step ranging from 1 to 2 hours. However, it is known that lentiviral vectors lose infectivity quickly at this temperature; indeed, the loss can be up to 40% of infectious units after 1 hour incubation at 37°C. Thus, we set to find out whether we could add Benzonase nuclease into the bioreactor during lentiviral vector production phase without affecting the production negatively, but while also maintaining Benzonase activity. First, to determine whether $MgCl_2$ should be added into the bioreactor for Benzonase activity and to assay any related toxicities, Benzonase digestion of plasmid DNA was carried out in DMEM both in the presence and absence of cells. Efficiency of the digestion was

assayed by Qubit dsDNA assay and ddPCR, while viability was assayed with NucleoCounter NC-3000. It was found that the 0.8 mM of $MgSO_4$ present in DMEM supported sufficient Benzonase activity, and no toxicities related to Benzonase or $MgCl_2$ were observed. The addition of Benzonase into the bioreactor 24h post-transfection was then successfully tested in the context of lentiviral vector production run in 10 m^2 scale-X carbo fixed-bed bioreactor, with a 95-fold reduction in dsDNA content at the 72h harvest point compared to a reference run with no Benzonase treatment. These findings contribute to an improved protocol for lentiviral production in a fixed-bed bioreactor.

P0477

Overview of CAR-T Cell Generation Using Optimized Plasmid Design and Lentiviral Vector Production

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Gene modified cell therapy such as CAR-T cells is one of the most promising advanced therapy medicinal products (ATMP) to fight against cancer. These therapies typically use lentiviral vectors to transfer a gene of interest and modify patient's cells. All the raw material used during the lentiviral vector manufacturing must be carefully selected based on different parameters such as quality, performance, supplier, etc. In this project, we generated CAR-T cells based on different plasmid constructs expressing a fusion protein CAR to track transduction efficiency. We first built different plasmid constructs using the plasmid engineering service from Polyplus, then encapsulate this gene of interest using a lentiviral vector produced with FectoVIR®-LV, transduce CD4+ CD8+ T cells from peripheral blood with this lentiviral vector, expand them for 12 days and finally test the efficiency of the transduced CAR-T cells using a killing assay.

P0478

TetraVecta™ Packaging Cell Lines for LV-CAR production

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HIV-1 based Lentiviral vector (LV) delivery of Chimeric Antigen Receptor (CAR) encoding transgenes is the industry standard for the generation of CAR-T cells for cancer immunotherapy. Derivation of an efficient clinical development pipeline and an economical manufacturing process are highly desirable for CAR-T therapeutic products. LV-CAR is typically generated via transient transfection of suspension HEK293T cells at 50-200L scale, although up to 2000L is now feasible. Stable producer cell lines (PCLs) offer a multitude of advantages for the manufacture of LVs in comparison to the standard transient transfection process. These include reduced production costs, scalability to large volumes, improved batch consistency and a more streamlined production

process. Transfection-based production lacks the lengthy cell line development timelines of PCLs and is sufficient to provide enough LV product for clinical trial purposes and consequently is typically progressed for commercial supply. Subsequent transition into PCLs-based LV production requires additional studies evaluating LV product comparability between both processes. Thus far, this late-stage hurdle has limited realisation of the benefits of PCLs.

The generation of high-titre LV-CAR PCLs has had limited success until recently, due to the constitutive expression of the CAR molecule negatively impacting production cell survival. Repression of the CAR transgene in production cells can be achieved using the TetraVecta™ System, developed by Oxford Biomedica (OXB), opening the pathway to LentiStable™ LV-CAR PCLs. Here, OXB highlights the use of off-the-shelf TetraVecta™ packaging cell lines (PaCLs) that could be utilised as a precursor to future PCL development. These PaCLs contain all necessary LV packaging components for the production of LV particles, as well as the TRiP System™ components that repress the transgene, allowing a flexible but simplified LV production process. We demonstrate robust LV-CAR productivity in TetraVecta™ PaCLs, achieving comparable or greater yields than the fully transient process, and successful scale-up into a 7L stirred-tank bioreactor. Following downstream processing, the concentrated and purified LV was capable of effectively transducing the relevant target cell type - human primary T-cells.

TetraVecta™ PaCLs offer an attractive alternative for the first step of LV-CAR product development by [1] reducing costs associated with GMP plasmid supply, [2] accelerating process development timelines, [3] improving batch-to-batch consistency, and [4] increasing comparability between LV product made using PaCLs and PCLs. Development of a PCL in parallel to clinical testing would enable a more straightforward transition into a fully transfection-free production process utilising the benefits of PCLs for commercial supply.

P0479

Preclinical validation of a novel lentiviral vector for treating neonatal inherited metabolic diseases

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Paediatric liver transplantation for inherited metabolic diseases (IMD) poses challenges such as survival rates, infection risk, and donor scarcity, necessitating lifelong medical surveillance. Gene therapy provides a potential alternative but faces obstacles such as depletion of vector DNA through hepatocellular proliferation, which reduces efficacy over time.

Lentiviral vectors (LV) have the potential to provide a one-time intervention as the payload integrates into the cell genome, meaning it is retained in progeny after cell division. However, integration can potentially cause safety problems, such as insertional mutagenesis. We have previously developed an optimized LV, LTR1, designed to mitigate safety risks via elimination of cis-regulatory elements in the lentiviral backbone.

We are developing gene therapy for Maple Syrup Urine Disease (MSUD), a severe IMD impacting Branched-Chain Amino Acid (BCAA) metabolism. Patients with MSUD can develop ataxia, motor delay, and intellectual disability due to amino acid and neurotransmitter imbalances and catabolic distress. Liver transplant has been validated for MSUD, as the liver is a hub for BCAA metabolism. We are investigating LTR1 liver gene therapy as a possible alternative to liver transplant.

We engineered an LTR1-based vector featuring a synthetic liver-specific promoter to facilitate the expression of a Dihydrolipoamide Branched-chain Trans-acylase (DBT) transgene in a mouse model of MSUD. Newborn MSUD pups received intravenous injections of the LTR1-*DBT* vector at doses of 1.4E11 transducing units per kilogram (TU/kg) (High-Dose, HD), 4.5E10 TU/kg (Mid-Dose, MD) or a four-fold lower dose (Low-Dose, LD) of the LTR1-*DBT* vector. Additionally, a third-generation LV expressing *DBT* under the control of a similar promoter was administered as an internal control.

Neonatal administration of the LTR1 vector significantly extended the lifespan and improved the body weight of MSUD mice across all dosage groups compared to untreated MSUD animals, which typically succumb within 25 days. Notably, the HD group showed 100% survival at four months when we terminated the study. Treatment with LTR1-*DBT* vectors resulted in a substantial, dose-dependent reduction in plasma BCAA concentrations. Untreated MSUD animals exhibited, on average, a 23-fold increase in BCAA:Alanine concentrations, whereas this ratio decreased to 3.2-fold in the MD group and 1.7-fold in the HD groups compared to age-matched wild-type controls. Although BCAA levels in LTR1-HD treated mice remained elevated, they resembled those observed in patients after liver transplantation.

Despite receiving similar doses of vector injection, LTR1-HD animals exhibited a significant decrease in BCAA levels compared to the experimental cohort treated with a third-generation lentiviral-based vector expressing *DBT*, where the BCAA: Alanine ratio was about 5 times higher than the wild-type values, highlighting the marked improvement in efficacy with the LTR1 vector.

Interestingly, animals treated with HD or MD of the LTR1-*DBT* gene therapy showed significant improvements in motor skills, locomotion, and hyperactivity, symptoms common in MSUD patients, whereas the LD group did not. This indicates that early neonatal gene therapy can alleviate neurological deficits associated with MSUD in a dose-dependent manner. The results demonstrate the potential efficacy of LTR1-based therapy in mitigating MSUD symptoms, highlighting its promise as a viable option to liver transplant.

P0480

Lenticlair™ 1: A Phase 1/2 trial evaluating the safety, tolerability and efficacy of an inhaled F/HN-pseudotyped lentiviral vector for CF gene therapy in people with CF ineligible for CFTR modulators

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BI 3720931, a third-generation lentiviral vector pseudotyped with Sendai virus F and HN envelope proteins expressing functional cystic fibrosis (CF) transmembrane conductance regulator (CFTR), has been developed as a gene therapy (GT) for CF. This first-in-human Phase 1/2 trial (Lenticlair™ 1) will evaluate the safety and efficacy of BI 3720931 in people with CF (pwCF) who are genetically ineligible for CFTR modulator therapy (CFTRmt).

The trial will enrol pwCF aged ≥18 years (males and females of non-childbearing potential) with a percent predicted forced expiratory volume in 1 second ($ppFEV_1$) between 50–100% at screening and stable disease with no recent exacerbations. Participants must be ineligible for CFTRmt and must either be naïve to prior GT or have a drug-free interval of at least 6 months for prior non-viral GT or at least 24 months for prior viral GT. Phase 1 is an open-label dose-escalation trial (n≥9) of a single low/medium/high dose of nebulised BI 3720931 (3/group) plus standard of care (SoC) with 24 weeks' follow-up (FU). The primary endpoint is the occurrence of drug-related adverse events (AEs) within 24 weeks. Secondary endpoints are the occurrence of treatment response defined as change from baseline ≥5% in $ppFEV_1$, the absolute change from baseline in $ppFEV_1$ at Week 24, and the occurrence of any dose-limiting toxicity up to Week 24. Bronchoscopy will be performed at Week 8 to quantify CFTR expression. Interim data from ≥8 weeks post-dose will inform Phase 2 dose selection. Phase 2 (n=27) is a randomised, double-blind, placebo-controlled, dose-expansion trial of a single dose of nebulised BI 3720931 (2 dose levels), or placebo, plus SoC (1:1:1) with 24 weeks' FU. The primary endpoint is the absolute change from baseline in $ppFEV_1$ at Week 8. Secondary endpoints are the absolute change from baseline in $ppFEV_1$ at Week 24, and the occurrence of any serious and drug-related AEs up to Week 24.

Results are pending. The trial is planned to begin in 2024.

The trial will evaluate safety and efficacy of BI 3720931 over 24 weeks in pwCF who are ineligible for CFTRmt.

P0481

Assessing the potential of a mutated HIV-1 protease for continuous stable lentiviral vector production

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Gene therapy has revolutionized modern medicine by providing treatment to previously unmet medical needs. The use of lentiviral vectors (LV) in gene therapy clinical trials and licensed products has been steadily increasing. While LV production traditionally relies on transient transfection, this method presents several disadvantages such as low scalability, high production costs associated with GMP-compliant plasmids, and a narrow production window due to the cytotoxicity induced by the LV components, namely, the LV protease and the vesicular stomatitis

virus G glycoprotein - VSV-G. Alternatively, LV production can be performed in stable systems such as constitutive producer cell lines, which are more cost-effective, scalable and standardized than transient productions. However, their development has been hindered by LV components-associated cytotoxicity. Strategies to mitigate this cytotoxicity include the use of non-cytotoxic gamma-retroviral envelope glycoproteins (e.g., Gibbon ape leukemia virus, GaLV, or the amphotropic murine leukemia virus, 4070A), and point-mutated variants of the protease like the T26S protease. However, the functionality of T26S is limited to stable production of 4070A pseudotyped LV, highlighting the need for novel protease variants with reduced cytotoxicity.

Herein, we assess the efficacy of a newly point-mutated protease (mutation X) for LV production and its potential for constitutive stable producer cell line development. This point-mutation is known to decrease the autoproteolytic activity of the protease without a major impact on K_{cat}/K_m compared to the wild-type (WT) protease. However, its impact on LV production and cell cytotoxicity remains unexplored. Herein, we inserted mutation X into the LV packaging plasmid and evaluated its functionality in both transient and semi-stable LV productions, using VSV-G, 4070A and GaLV for pseudotyping. The WT gag-pro-pol (GPP), GP(D25N)P (inactive protease) and GP(T26S)P (less active protease) were used as comparison.

Transient productions showed that the GP(X)P supported total particles (T.P.) and transducing units (T.U.) titers similar to those of WT GPP. This was found to be independent of the pseudotype suggesting that mutation X does not have a major impact on proteolytic processing and LV maturation.

To explore its potential for stable LV production, cells were stably transfected with GP(X)P, as well as the control GPP variants. Following stable selection, REV was also integrated by stable transfection. These cell populations were characterized for T.P. and T.U. in semi-stable productions (transgene and envelope glycoproteins were delivered via transient transfection). Results showed that cells stably expressing GP(X)P supported T.P. titers similar to those expressing the GP(T26S)P. Most importantly, cells stably expressing GP(X)P were able to successfully produce functional T.U. pseudotyped with GaLV, contrarily to GP(T26S)P cells. Furthermore, GP(X)P cells exhibited a higher cell growth rate than those expressing the WT GPP, despite having similar gag-pol expression levels. These findings suggest that this mutation may result in a less cytotoxic lentiviral GPP variant, supporting its use for further stable producer cell line development.

In conclusion, this study demonstrates the functionality of a novel mutated GPP variant for transient LV production and highlights its potential for the development of continuous stable LV producer cell lines with clinically relevant pseudotypes.

P0482

Gene Therapy for Adult and Pediatric Patients with Severe Pyruvate Kinase Deficiency: Results from a Global Study of RP-L301

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Pyruvate kinase deficiency (PKD) is a rare hemolytic anemia caused by *PKLR* gene mutations. Manifestations include anemia, splenomegaly and iron overload, which may be life-threatening. Current treatments are limited to chronic blood transfusions, iron chelation and splenectomy, all associated with significant side effects, or PK activators. A Phase 1 trial (NCT04105166) is evaluating lentiviral (LV)-mediated hematopoietic stem and progenitor cell (HSPC)-targeted gene therapy (RP-L301) in splenectomized patients with severe PKD (hemoglobin [Hb] < 8g/dL and/or transfusion-dependent anemia).

Following apheresis, HSPCs were transduced with LV vector and cryopreserved. Myeloablative therapeutic drug monitoring-guided busulfan was administered prior to RP-L301 intravenous infusion. Follow-up was 2 years to assess safety (including insertion site analysis [ISA]) and efficacy (genetic correction, transfusion requirements, anemia and hemolysis markers), with a subsequent long-term follow-up study.

As of February 2024, two adult and two pediatric patients have received RP-L301. All achieved neutrophil engraftment by day 15. Genetic correction (peripheral blood vector copy number up to 2.0) was observed in all patients. All patients have had clinically significant hemoglobin improvement of ≥1.5 g/dL with 3 of 4 patients demonstrating hemoglobin normalization up to the latest follow-up visit (up to 36 months). No patients required transfusions up to the latest follow-up visit (up to 36 months). Concurrent improvement in hemolysis markers including bilirubin and reticulocytes have been observed. Both adult patients reported improved quality of life (increased Functional Assessment of Cancer Therapy-Anemia and 36-Item Short-Form [SF-36] health survey scores, with marked improvement in SF-36 energy/fatigue, physical functioning, and general health domains). Pediatric quality of life assessment is ongoing. No serious adverse events are attributed to RP-L301. Peripheral blood ISA up to 36 months of follow-up indicate highly polyclonal patterns; longitudinal results delineating clonal diversity will be presented.

RP-L301 is a potential treatment for patients with severe PKD, including those who did not derive benefit from available therapies (including splenectomy and mitapivat). Robust and sustained efficacy up to the latest follow-up visit (up to 36 months post-treatment) is demonstrated by clinically significant improvement in hemoglobin and absence of transfusion requirements.

P0483

Clinical Readiness of PD-L1 Hematopoietic Gene Therapy for Type 1 Diabetes

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Type 1 diabetes (T1D) is an autoimmune disease characterized by a progressive destruction of insulin producing b-cells mainly conducted by auto-reactive T cells that have escaped central and peripheral immune tolerance. Current treatments for T1D are non-curative mostly because of lack of specificity and need for chronic and specialised medical management, exposing patients to serious and long-lasting side effects.

Several observations highlight the importance of the immune-regulatory factor programmed death-ligand 1 (PD-L1) and its receptor (PD-1) inhibitory pathway in the maintenance of immune homeostasis and tolerance. Preclinical studies along with data on T1D patients showed defective expression of PD-L1 on the hematopoietic stem/progenitor cell (HSPC) compartment.

In this context, we demonstrated in a pivotal Proof of concept (PoC) study that transplantation of HSPCs genetically engineered to overexpress PD-L1 can reverse hyperglycaemia in non-obese diabetic (NOD) T1D animals upon selectively trafficking to the inflamed pancreas and abrogation of the autoimmune response, as shown also by reduced islet T cell infiltration and preserved endogenous insulin release. This effect was achieved through the induction of apoptosis in autoreactive CD4+ and CD8+ T cells, mediated by genetically engineered murine HSPCs overexpressing PD-L1. Importantly, albeit these results demonstrate that the induced expression of PD-L1 in HSPCs may be used as a tool for targeted immunotherapy in T1D, this approach resulted particularly beneficial in stably normalizing glycaemia in mice having a residual β-cell function at time of the transplant, providing key indications for the identification of the optimal target population that could benefit from this approach.

Here, we developed the PD-L1 HSPC-based gene therapy approach for T1D and advanced it to clinical readiness. We developed a clinical-compliant third generation lentiviral vector (LV) encoding a codon-optimized human PD-L1 cDNA and tested it into a PoC of efficacy study in NOD mice. In these conditions we further extended the benefits associated to PD-L1 overexpression, particularly in terms of the timing of reversal to normoglycemia.

Then, to allow clinical translation of the PD-L1 gene therapy strategy, we employed a novel and highly effective protocol to transduce at high efficiency in large scale and clinically compliant conditions bona fide long-term human repopulating HSCs and tested the functionality, biodistribution, persistence, safety and tumorigenicity of these cells in two pivotal studies conducted in an immunodeficient mouse strain, one of which under Good Laboratory Practice (GLP).

Safety concerns were also investigated as required for transition to clinical testing and application.

Overall, preclinical data on NOD animal model along with toxicology and safety read out on immunodeficient mice provided strong PoC and safety indication for the clinical translation of PD-L1 gene therapy approach for the treatment of T1D.

P0484

Novel Cyclosporine analogues enhance transduction efficiency in human stem cells via IFITM3 degradation

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Allogenic stem cell gene therapy is a life-changing treatment for a variety of disorders including children with severe combined immunodeficiency. These treatments typically use HIV-based lentiviral vectors pseudotyped with a VSV-G envelope to deliver therapeutic genes to stem cells *ex vivo*. A key limitation is poor transduction efficiency on primary human stem cells (HSPC). It was previously shown that a key factor defining HSPC permissiveness is the expression of the anti-viral restriction factor IFITM3. Fortunately, treatment with Cyclosporines leads to IFITM3 degradation and enhances LV infection in stem cells. In this study we have used structure-aided design to chemically modify Cyclosporine A to eliminate unwanted Cyclophillin A targeting and enhance IFITM3 targeting. Thus, we have created a novel non-immunosuppressive cyclosporine analogue, CycloVect, that mediates IFITM3 degradation and potently enhances LV transduction of HSPC. Mechanistic studies indicate that CycloVect disturbs normal IFITM3 recycling, rerouting it to be degraded in lysozymes. Thus, CycloVect works downstream from, and additively with, current gold standard transduction enhancers that promote LV attachment such as protamine sulphate. By identifying IFITM3 restriction as key block to lentiviral transduction of HSCs we have developed a targeted transduction enhancer comparable in efficacy to current gold standard treatments such as Lentiboost. Expression levels of IFITM3 and other constitutively active ISGs could serve as biomarkers for predicting transduction efficiency, with a view to help reduce variability in patient outcomes.

P0485

Unravelling the effect of proliferative stress and genotoxicity in hematopoietic stem cells in vivo

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Hematopoietic Stem and Progenitor Cells (HSPCs) from patients affected by inherited disorders can be corrected with the use of Gene Therapy (GT), providing long-term therapeutic benefit upon reconstitution of the entire hematopoietic system. However, vector insertions may result in oncogene activation or tumour suppressor inactivation, resulting in malignant transformation as recently shown in the lentiviral vector (LV) based clinical trial for adrenoleukodystrophy and/or clonal expansions in different cancer immunotherapy applications. Moreover, the replicative stress accumulated during hematopoietic reconstitution and aging, may induce cellular senescence and trigger a chronic inflammatory response leading to hematopoietic decay.

Here, to address the impact of insertional mutagenesis and age on haematopoiesis, we studied the clonal dynamics of hematopoietic reconstitution over time in wild-type (WT) C57 mice transplanted with HSPCs transduced either with a genotoxic LV harbouring the strong retroviral enhancer/promoter Spleen Focus Forming Virus in the Long Terminal Repeats (LTR), or with a safer self-inactivating LTRs and the moderate cellular promoter PGK. Additionally, the same HSPC-GT strategy was applied by transplanting *Cdkn2a*$^{-/-}$ HSPCs, which lack p16^{INK4A} and p19^{ARF} proteins and thus have no barriers against protooncogene activation. Blood composition and vector integration sites (IS) of B, T, and myeloid cells were monitored overtime (up to 2.5 years). Somatic mutations were identified by analysing the genomic portion of the mouse flanking each IS, and to assess mutation accumulation rates we developed a new Mutation Index (MI) which normalizes the number of mutations by clones and coverage.

As expected, vector-driven activation of the *Braf* proto-oncogene prompted the acceleration of myeloid tumour onset in the *Cdkn2a*$^{-/-}$/genotoxic group compared to the *Cdkn2a*$^{-/-}$/non-genotoxic group ($p<0.0001$). On the contrary, none of mice receiving WT cells developed tumours. Analysis of the blood composition overtime showed an age dependent skewing towards the myeloid lineage in all mice, which was exacerbated in both groups of mice transplanted with HSPCs transduced with the genotoxic LV. Overall, we retrieved >200,000 IS, corresponding to 9 Gb of sequence genomic information. We found that the MI in both the groups receiving HSPCs transplanted with the genotoxic LV were significantly higher when compared to non-genotoxic groups ($p<0.001$) and specifically myeloid clones exhibited a higher mutation frequency compared to B and T cell lineages. Correctly reflecting that the presence of genotoxic insertion resulted in progressive somatic mutation accumulation and insertional mutagenesis. Moreover, the MI of the mice receiving WT HSPCs transduced with the genotoxic LV in the myeloid compartment was significantly higher than mice receiving *Cdkn2a*$^{-/-}$ cells transduced with the same vector ($p<0.01$), reflecting the effect of aging.

These results demonstrate for the first time that vector genotoxicity not only increases the risk of oncogenic transformation but has also a negative impact on haematopoiesis by triggering accelerated aging of the hematopoietic system accompanied by accumulation of somatic mutations across hematopoietic lineages in vivo.

P0486

Lenticlair™-ON: An extension trial examining long-term safety and efficacy outcomes associated with an inhaled F/HN-pseudotyped lentiviral vector for CF gene therapy in people with CF

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BI 3720931, a third-generation lentiviral vector pseudotyped with Sendai virus F and HN envelope proteins expressing functional cystic fibrosis transmembrane conductance regulator (CFTR), has been developed as a gene therapy (GT) for cystic fibrosis (CF). BI 3720931 will be assessed in a first-in-human (FiH) Phase I/II trial (Lenticlair™ 1) in people with CF (pwCF) who are genetically ineligible for CFTR modulator therapy. Following completion of Lenticlair™ 1, all trial participants will enter an extension trial for long-term follow-up (FU; 15 years, as per regulatory guidelines) of safety and efficacy (Lenticlair™-ON).

The primary endpoint is the occurrence of new serious adverse events (SAEs) up to 15 years post-enrolment, including risks outlined by regulatory guidance documents for integrating GTs. Secondary efficacy endpoints are the time to loss of efficacy (drop to <5% above individual baseline in percent predicted forced expiratory volume in 1 second [ppFEV$_1$]), and the time to first pulmonary exacerbation from dosing. Secondary safety endpoints are the occurrence of adverse events up to 2 years after enrolment, and the occurrence of SAEs and replication-competent lentivirus throughout the trial. Interim analyses will take place at the end of the FiH trial and then every 2 years. Long-term FU will involve quarterly visits during Years 1–2, followed by annual visits during Years 3–15.

Results are pending. This extension trial will allow seamless rollover of the first participant from the FiH Lenticlair™ 1 trial.

This trial will investigate long-term safety and the duration of efficacy in pwCF treated with BI 3720931.

P0487

Development of a Third-Generation Lentiviral Vector for GLP-1 Gene Therapy: Enhancing β-Cell Function and Mass in Type 2 Diabetes

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Type 2 diabetes (T2D) presents a significant global health challenge, characterized by insulin resistance and impaired pancreatic β-cell function. Despite advancements in treatment, achieving glycemic control and managing related complications remain challenging. Glucagon-like peptide-1 (GLP-1) has emerged as a promising treatment for T2D due to its diverse effects on glucose metabolism, β-cell function, and weight regulation. GLP-1 enhances glucose-dependent insulin secretion, reduces glucagon secretion, and slows gastric emptying. Beyond immediate effects,

GLP-1 promotes β-cell growth and preserves β-cell mass, offering potential long-term benefits. However, reduced GLP-1 secretion in T2D underscores the importance of strategies to protect β-cells and increase their mass. Numerous studies on GLP-1 analogs and mimetics have shown promising therapeutic profiles but are limited by side effects and the necessity for daily injections. Consequently, new treatment strategies using the natural form of GLP-1 to protect and increase β-cell mass are needed. Gene therapy emerges as a promising approach, targeting the fundamental genetic causes of the disease, potentially offering long-term or permanent solutions, and reducing the need for continuous medication and management. Among gene delivery methods, lentiviral vectors are notable for their efficient gene integration and broad cell tropism, making them ideal for delivering therapeutic genes like GLP-1. In this study, a novel HIV-based third-generation lentiviral vector (LentiGLP-1) was engineered to synthesize GLP-1 under the control of the CMV promoter. After production, a series of *in vitro* experiments were conducted, including titration analysis, GLP-1 enzyme-linked immunosorbent assay, MTT growth assay, and glucose-stimulated insulin secretion test. Results showed that LentiGLP-1-infected beta cells synthesize and secrete GLP-1, with a notable increase in insulin secretion at high glucose concentrations, indicating the presence of functional GLP-1 peptide with incretin properties. Promising results from *in vivo* experiments suggest further investigations in diabetic animal models to explore LentiGLP-1's potential in preserving β-cell mass. This study advocates for exploring GLP-1 function on pancreatic endocrine cell differentiation in an STZ-induced neonatal experimental animal model of diabetes through dual immunohistochemical analyses with various pancreatic differentiation, neogenesis, and proliferation markers. *(TUSEB Grant No: 22509)*

P0488

Harnessing Chromatin Architecture and Post-Transcriptional Regulation to Benefit the Safety of the Lentiviral Vector Platform

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Self-Inactivating Lentiviral Vectors (SIN.LVs) are widely used in gene therapy to integrate therapeutic transgenes into patient cells' genomes to cure a variety of diseases. While demonstrating notable efficacy and safety in preclinical and clinical settings, genotoxicity mediated by LV integration has been reported in Hematopoietic Stem/Progenitor Cell gene therapy and in Chimeric Antigen Receptor (CAR) T cell cancer immunotherapy trials. While the risk-benefit valuation of these therapies remains positive, with significant benefits for patients, these events underscore the need for mitigating potential risks associated with vector integration. Here, we devised a strategy to overcome activating insertional mutagenesis events by leveraging i) chromatin insulators (CI) to reduce the interactions between the vector enhancers and cellular promoters, and ii) sequences complementary to micro-RNA (miRT), to trigger degradation of oncogenic aberrant/chimeric transcripts. To test our strategy, we used an in vivo assay of liver oncogenesis sensitive to LV-mediated insertional mutagenesis, where neonatal WT mice are injected with LVs and treated with CCl4 cancer promoting agent. Mice are euthanized after 1-year of follow-up to collect tissues for histopathology and integration sites analysis (ISA) to define the molecular culprits of oncogenesis. We generated a LVs SIN LTRs with the liver-specific Enhanced Transthyretin (ET) enhancer-promoter in internal position driving GFP expression. To this reference LV, we included liver-active and hematopoietic specific miRT, to degrade aberrant transcripts from both tissues, and CI. As CI operate by forming chromatin loops between

convergently oriented sites, we cloned these elements in convergent or divergent orientations in the LV, to shield host genes either by confining the ET-enhancer in a LV-internal loop or by excluding the enhancer from external loops mediated by genomic CI. An active ET LTR LV was used as genotoxic control. Histopathological analysis identified 34 liver tumors (out of 30 mice) from the genotoxic ET.LTR LV group and 14 masses (out of 27 mice) from SIN.AS LV treated mice. Differently, the LVs armed with CIs and miRTs showed a significantly lower incidence of liver tumors, with only 3 and 1 tumors (out of 34 and 28 mice) respectively, indicating a superior safety profile. This observation was confirmed by ISA on DNA from liver derived masses. In the ET.LTR group, we observed robust targeting of Rlt1-Rian locus, a well know player of insertional mutagenesis in this model. In liver masses marked by SIN.AS LV, fewer Rtl1-Rian integrations were present, and we observed the emergence of clones with integration targeting Lingo2, Cchcr1 and Diap3, whose upregulation might be involved in proliferation and transformation. Differently, mice treated with the LVs carrying CI and miRT, did not show targeting of Rtl1- Rian locus and we found one marked tumor with an insertion in Erh gene, which has a reported role in proliferation. Overall, the reduced frequency of tumors induced by the safety-improved LVs and the absent targeting of genes commonly found in liver genotoxicity indicate that the combination of miRT and CI allows to improve the safety of the LV platform.

P0489

Assessment of safety and efficacy of *in vivo* lentiviral gene therapy for ARC syndrome

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Arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome is a severe autosomal recessive multi-system disorder resulting from mutations in the *VPS33B* gene. The deficiency of *VPS33B* in hepatocytes leads to hepatocyte apical membrane dysfunction, causing progressive liver fibrosis and end stage liver disease. Research utilizing animal models suggests that lentiviral vectors may offer a safe option for *in vivo* gene therapy applications, facilitating sustained transgene expression. Our aim was to develop a lentiviral-based gene therapy to treat ARC syndrome. To confirm the safety of this approach, we have developed two lentiviral vectors LV.EF1α.co*VPS33B* (ubiquitous) and LV.LP1.co*VPS33B* (liver specific) and tested them in heterozygous *Vps33b*$^{+/-}$ knockout mice, which do not exhibit the ARC phenotype. The neonatally treated mice were analysed at 9 months. Our results have raised safety concerns for the systemic delivery of the LV.EF1α.co*VPS33B* vector, with 5:10 treated mice developing liver abnormalities (including 3 hepatocellular carcinomas) compared to 0:10 of the mice treated with the LP1 containing vectors. Integration site analysis from tumour samples revealed a decreased Shannon diversity score, a low integration site diversity and identified *Tox1* as a common integration locus between the 3 hepatocellular carcinomas. Additionally, transcriptomic data has also revealed that the expression of 8371 genes has been dysregulated in the tumour samples compared to healthy liver tissues. Following the confirmation of safety for LV.LP1.*coVPS33B* treatments, we further investigated their efficacy in addressing the ARC liver phenotype in neonate *Vps33b*$^{-/-}$ knockout

mice. The therapeutic effect was assessed 12 weeks post-treatment, after the mice had been fed a 0.25% cholic acid diet for 8 weeks. The results have shown improved survival, growth as well as normalisation to wild-type values of ARC syndrome specific blood parameters such as alkaline phosphatase and total cholesterol. Histopathological analysis revealed a reduction in the amount of liver fibrosis and number of immune cells infiltrates. Immunofluorescence microscopy and correlative light-electron microscopy have shown restoration of the bile canalicular ultrastructure. These findings offer hope for ARC syndrome patients and underscore the importance of rigorous safety screening for newly developed lentiviral vectors.

P0490

Preventing telomeric and extratelomeric hallmarks of dyskeratosis congenita in X-linked DC-like CD34+ cells by lentiviral-mediated gene therapy

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The dysregulation of the telomere maintenance is the underlying cause of telomere biology disorders (TBDs), characterized by accelerated telomere attrition, limited cellular replicative potential and hampered tissue regeneration. Dyskeratosis congenita (DC) is a life-threatening telomeropathy, which in 80% of the patients is associated with progressive bone marrow failure (BMF). X-linked DC (XDC) arises from germline mutations in the *DKC1* gene. Currently, allogeneic hematopoietic stem cell (HSC) transplantation constitutes the only curative option to correct BMF in these patients; however, alternative therapeutic approaches are necessary due to the limited availability of compatible donors and the toxicity associated to this therapeutic modality.
Since *DKC1* is constitutively expressed in different human cell types, here we aimed at evaluating the efficacy of a lentiviral vector (LV)-mediated gene therapy approach to correct the phenotypic hallmarks of XDC HSCs. With this purpose we generated two LVs that carried the *DKC1* gene under the regulation of different regulatory sequences. To determine the efficacy of these LVs to correct the phenotype of human XDC HSCs, XDC-like HSCs were developed due to the very limited availability of BM samples from XDC patients. Briefly, knock-out *DKC1* mutations and also a hypomorphic Thr66/Ala missense (c.196 A>G SNP) mutation were introduced in healthy donor cord blood CD34+ cells using specific gRNA sequences and AAVs that harboured the *DKC1* hypomorphic mutation. Cells harbouring *DKC1* mutations, particularly those harbouring KO mutations showed marked proliferation defects compared with HD or with cells carrying the hypomorphic mutation. Additionally, XDC-like CD34+ cells exhibited an exacerbated telomeric attrition and a pseudouridine synthase defective activity compared to control, non-edited cells. To evaluate whether the therapeutic LVs corrected the phenotypic defects of XDC-like HSCs, *DKC1*-mutated CD34+ cells were then transduced either with an EGFP-LV that was used as a control LV, or with each of the therapeutic vectors. In contrast with data obtained in EGFP-transduced XDC-like cells, the transduction with dyskerin therapeutic vectors preserved the telomeric length and enhanced the *ex vivo* expansion of XDC-like HSCs. Furthermore, these vectors corrected the defective pseudouridine synthase activity observed in XDC-like CD34+ cells. Ongoing *in*

vivo studies aim to confirm the long-term efficacy and safety of LV-mediated gene therapy in XDC-like human HSCs.

P0491

Gene therapy corrects adenosine deaminase 2 deficiency in hematopoietic stem progenitor cells without altering their characteristics

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Loss-of-function mutations in the adenosine deaminase 2 (ADA2) gene cause DADA2, an inherited immune disorder characterised by vasculopathy and immunological and haematological defects, often progressing to bone marrow (BM) aplasia. Current treatments include life-long anti-TNF therapy, while haematopoietic stem and progenitor cell (HSPC) transplantation is reserved for severe cases. To evaluate the potential of HSPC gene therapy as an effective treatment for HSPC impairment, we developed a third-generation lentiviral vector (LV) expressing a functional ADA2 gene. Transduction of HSPCs from DADA2 patients successfully delivered functional ADA2, as confirmed by Western blot and extracellular ADA2 activity assay. The vector copy numbers (VCNs) in transduced patient HSPCs were comparable to those in healthy donor (HD) HSPCs. Colony-forming unit (CFU) and in vitro differentiation assays were conducted to evaluate the clonogenic and multilineage differentiation potential of transduced patients' HSPCs. The CFU assay demonstrated high transduction efficiency and VCN across various primitive cell lineages, with no toxicity in patients' HSPCs. Both CFU and in vitro differentiation assays revealed no significant alterations in the clonogenic and differentiation potential of ADA2-transduced DADA2 HSPCs compared to untransduced cells. In vivo studies in immunodeficient mice revealed successful engraftment and reconstitution of the complete haematopoietic lineages by transduced DADA2 patient HSPCs. Integration site analysis revealed a polyclonal integration pattern and confirmed the absence of genotoxicity.

Our findings demonstrate that ADA2 expression and activity were effectively restored in patients' HSPCs, which maintain their clonogenic and differentiation capabilities. Additionally, preclinical studies in mice confirmed the safety and tolerability of ADA2 expression in patients' HSPCs. The successful in vivo engraftment of ADA2-corrected HSPCs highlights the promising therapeutic potential of gene therapy for treating DADA2.

P0492

Administration of lentiviral vector doses achieving high transduction efficiency is well tolerated in mice

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We have developed a lentiviral vector pseudotyped with the Sendai virus F and HN envelope proteins (rSIV.F/HN) to deliver a normal copy of cystic fibrosis transmembrane conductance regulator (CFTR) complimentary DNA into the genomic DNA of airway epithelial cells of people with cystic fibrosis (pwCF) (BI 3720931). Here, we assessed the tolerability of rSIV.F/HN at doses that achieve high transgene expression in mice.

C57BL/6 mice (36 males and 36 females) received once-daily doses of the rSIV.F/HN vector expressing enhanced green fluorescent protein (EGFP) ($2x10^8$ transduction units (TU)/dose) or diluent for 4 consecutive days via intranasal instillation (total dose of $8x10^8$ TU/mouse). On Days 2 or 7 after the last dose, mice were euthanised (9 of each sex/group/day). Body weight, blood (for clinical pathology and cytokine analyses) and lung tissue samples (for histopathology, immunohistochemistry, quantitative polymerase chain reaction (qPCR) and reverse transcription qPCR analyses) were collected.

Clinical observations and survival were favourable. Increases in body weight 7 days after the last dose were lower (0.1 g, n=8) in vector-treated female mice than in controls (0.8 g, n=9). Some inflammatory cytokines were modestly elevated (RANTES, MCP-1, TNFα) or decreased (VEGF, MIP-1α) at one or both time points. Microscopic examination of lung tissue 2 days after the last dose indicated non-adverse, minimal-to-mild perivascular-to-peribronchovascular infiltrates of primarily mononuclear cells, which became minimal in severity 7 days after the last dose. Other than minimally lower calcium in vector-treated animals of both sexes at 2 and 7 days after the last dose (reason unknown), no other biologically relevant changes in clinical pathology were observed. A mean of ~35% ± SD 5.7 of airway epithelial cells expressed EGFP. Integrated vector genome DNA was detectable in approximately 7% of lung epithelial cells and levels of vector-specific messenger RNA (mRNA) were more than 3 log orders higher than endogenous CFTR mRNA levels.

These data demonstrate that rSIV.F/HN transduction of mouse lungs is well tolerated at doses that achieved significant transduction levels. These data, together with other preclinical data, support further progression of BI 3720931 towards the clinic. A first-in-human, Phase 1/2 trial (Lenticlair™ 1) to evaluate the safety and efficacy of BI 3720931 in pwCF who are genetically ineligible for CFTR modulator therapy is planned to begin in 2024.

P0493

IVIM assay for retroviral vector induced-genotoxicity: insights on 15 years of research

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Retroviral vector-mediated gene therapy has shown clear clinical benefit as a treatment option for various diseases. While integrating vectors provide life-long correction of genetic disorders, they can also trigger malignant cell transformation by insertional dysregulation of surrounding genes. Severe adverse events in some clinical trials induced by such technologies urge the field for a more reliable pre-clinical safety assessment. Amongst the few available tests, the *In Vitro* Immortalization Assay (IVIM) is actively requested by regulatory agencies and companies prior to clinical translation of different vector designs. IVIM is based on the premise that murine hematopoietic stem and progenitor cells transduced with mutagenic vectors can acquire a proliferation advantage under limiting dilution conditions, unlike untransduced controls, which usually cannot survive. The classical readout combines a microscopic screening of outgrowing clones, as the most evident sign of immortalization, and an MTT assay for metabolically active cells. However, setting a proper threshold for the MTT scoring can be challenging, especially when untransduced cultures display spontaneous background proliferation. An extensive analysis of the data acquired over the last 15 years has shown the microscopic scoring to be reliable enough as a readout for genotoxicity, leaving the need for performing an MTT obsolete, thus reducing costs and assay times. Here, we present a comprehensive meta-data analysis of previous assays and the current cut-off strategy for genotoxicity scoring with the IVIM assay.

P0494

Variable sensitivity of lentiviral vectors to IFITM restriction factors is observed with different glycoprotein pseudotypes and may be overcome by transduction adjuvants

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Gene therapy is an attractive option to cure an ever-increasing number of pathologies. Among different tools that have been developed in previous years, lentiviral vectors derived from the infectious HIV-1 virus have proved particularly versatile and useful tools for gene transfer or for gene editing to treat a series of diseases with gene-modified hematopoietic stem cells or with engineered T cells. Major improvements in strategies and viral vector design have facilitated lentiviral vector usage. In particular, the use of various pseudotypes is a constantly evolving domain to enable the more precise transduction of specific cell types in vitro or in vivo. Despite all these efforts, current lentiviral vectors remain particularly sensitive to several innate immune

proteins, called restriction factors, that severely restrict the transduction efficiency of specific cell lineages. Here, using overexpressing model cell lines, we investigated the sensitivity of lentiviral vectors to IFITM restriction factors depending on the nature of the glycoprotein used for pseudotyping. Vectors pseudotyped with glycoproteins from vesiculoviruses showed an overall good resistance to the effects of IFITMs proteins, and were not strongly affected by the transduction adjuvants that were tested. Lentiviral vectors pseudotyped with glycoproteins of retroviral origin showed a much higher variability in their sensitivity to IFITM proteins, suggestive of a strongly reduced efficacy for clinical applications on specific target cells. Interestingly, the observed reduced infectivity can be counteracted to some extent by cyclosporin H, but also unexpectedly by Vectofusin, both of which were among the tested adjuvants. These results confirm the interest of adapting the lentiviral vectors pseudotyping not only to each application, but also to the transduction conditions and taking into account the IFITM expression levels. Together, these parameters can strongly enhance the general efficacy of the transduction, especially for those target cells that naturally express proteins of the IFITM family, including the human hematopoietic stem/progenitor CD34+ cells that are of particular clinical interest to treat immune or blood disorders.

P0495

Development of a lentiviral vector-based gene therapy for the treatment of Major Histocompatibility Complex Class II deficiency (MHCII-D) in patients with mutations in RFXANK gene

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MHC-II deficiency (MHCII-D) is a rare and severe combined inborn error of immunity following an autosomal recessive inheritance pattern. It is caused by mutations in genes encoding for proteins involved in the transcription of the MHC-II locus. Among patients identified worldwide with MHCII-D, approximately two-thirds have a genetic defect in RFXANK gene, with most families being of North-African origin and sharing the same founder mutation.

Hematopoietic stem cell transplantation (HSCT) is the only available curative treatment, however partial results have been reported in the literature, especially in the absence of compatible donors. Gene therapy arises as a promising alternative to HSCT, where autologous HSCs are corrected and reinfused to the patients, sorting out difficulties related with allogenic transplantation.

The aim of the project is to develop and characterize a gene therapy approach based on ex-vivo correction of RFXANK defect in patients' HSCs, using a lentiviral vector.

A functional lentiviral vector (LVV) for RFXANK has been generated. The sequence corresponding to RFXANK cDNA (of Homo sapiens) was synthesized and cloned into a 3rd generation lentiviral

plasmid (pCCL) under the control of PGK promoter. Lentiviral vector particles were produced using HEK293T cell line and a third-generation system (4 plasmids).

To test transgene functionality, complementation assays were conducted on BLS-1 cell line (B-LCL) generated from cells of patients carrying the founder mutation in RFXANK gene.

In order to obtain the relevant target cells (CD34+), an RFXANK-/- CD34+ cell model was generated by CRISPR/CAS9 gene editing from healthy CD34+ cells.

Complementation assays demonstrated that: (i) RFXANK correction restores MHC-II expression on the cell surface; (ii) RFXANK protein is correctly localized in the nucleus of the cells; and (iii) overexpression of RFXANK does not lead to an increased expression of MHC-II. Importantly, results suggest that a tight control over RFXANK expression level may not be required for the gene therapy approach, since other factors, such as other members of the RFX complex or CIITA, act as limiting factors for MHC-II expression.

A transduction protocol of CD34+ cells with the lentiviral vector was optimized, by testing different cell culture and transduction conditions. Efficiency and safety parameters, such as transduction efficiency and vector copy number, are under evaluation.

Results of these studies will provide the best conditions to generate the first gene therapy-based approach for MHC-II deficiency, using a lentiviral vector. Manufacturing conditions and reagents that comply with the principles of GMP guidelines for cell and gene therapies will be used.

P0496

Developing stem cell and gene therapy for VPS33B deficiency

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Arthrogryposis Renal dysfunction and Cholestasis syndrome (ARC) is an autosomal recessive multisystem disorder. Around 75% of ARC cases are due to mutations in *VPS33B* encoding VPS33B that forms a stable cytoplasmic complex (CHEVI) with VIPAR (encoded by *VIPAS39*). CHEVI regulates protein trafficking and biosynthesis of specialised organelles. Most patients die of severe infections or bleeding before 18 months of age. Bleeding defects in ARC result from abnormal platelet α-granules synthesis in megakaryocytes. Infections in ARC may be due to a defect in phagosome-lysosome and endosome-lysosome fusions in macrophages and other immune cells. Platelet α-granule deficiency and immune dysregulation are reported to be associated with VPS33B deficiency. The aim of this study was developing an *ex vivo* gene therapy to prevent bleeding and infections which are counted as the fatal consequence of ARC. Over the last decades, *ex vivo* lentiviral gene therapy of haematopoietic stem cells (HSCs) has shown promise in humans and animal models of genetic disorders affecting immune and haematological systems. We have developed a lentiviral vector LV.EF1α.co*VPS33B* and tested it in a tamoxifen inducible ERT^{2}-Cre-*Vps33b*$^{fl/fl}$ mouse model. When induced post developmentally, this mouse model displays the bone marrow abnormalities such as abnormal platelets, mimicking those

observed in ARC patients. A week after inducing *Vps33b* knockout, some of the knockout mice were sacrificed as bone marrow donors. HSCs isolated from the bone marrow were transduced with LV.EF1α.co*VPS33B* and injected intravenously into surviving tamoxifen induced ERT^{2}-Cre-*Vps33b*$^{fl/fl}$ mice after irradiation to secure engraftment. Overall, the treated mice tolerated the procedure well; a 6-fold increase in the expression of co*VPS33B* was detected in treated mice compared to the wild type mice with an average of 2.5 vector copies per cell. The treated mice demonstrated improvements including reduced spleen size and increased survival for the first time, providing proof of concept for this treatment approach in ARC syndrome.

P0497

Insertion site profile analysis of lentiviral vector transduced ARI-0001 (autologous CAR19-T cells) infusion products

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Characterization of the insertion-site profile of viral vector modified gene therapies is important to monitor the safety of the products and anticipate and/or detect any possible genotoxic events due to insertional mutagenesis. This analysis is critical for therapies targeting hematopoietic stem cells (HSC) cells, since the modified cells will be long-lived in the patient's body. The first evidence of secondary neoplasms due to insertional mutagenesis came from the first clinical trials using γ-retroviral vector-modified cells, in X-SCID, Wiskott-Aldrich and Chronic Granoulomatous Disease patients. Since then, several strategies have been used to improve the safety of viral vectors. Indeed, γ-retroviral vectors have extensively been replaced by lentiviral vectors in gene therapy approaches. Lentiviral vectors have been shown to differ in the integration-site profile, compared to γ -retroviral vectors. While the latter seem to preferentially integrate around transcription-start sites (TSS), lentiviral vectors preferentially integrate in transcription units and gene-dense regions.

Since the first clinical trial using a Chimeric antigen receptor (CAR)-T cell product for the treatment of hematological malignancies, the number of patients receiving a gene therapy product has increased exponentially. Although T-cells are differentiated cells and in most cases, short-lived, in some patients the modified T-cells can remain in the body for years or even decades. Although, overall, CAR-T cells have been proven safe, as the number of treated patients increases, some cases of secondary malignancies have been reported. The potential connection of these secondary malignancies to insertional mutagenesis events is still controversial. However, the need to characterize site-integration profile in these products has been reinforced.

Here, we present the results of site-integration analysis of ARI-0001 products (an autologous, lentiviral-vector transduced, anti-CD19-based CAR). Quantitative Shearing Linear Amplification Mediated PCR (qs-LAM-PCR) has been used to identify and quantitate each insertion site and clonal frequency in infusion products. No evidence of clonal insertional events have been found. A strong preference for intragenic regions is clearly observed, although about 93% of these insertions occur within introns, reducing its mutagenic potential. Only a residual percentage of total insertions have been identified in exons or UTR regions. Also, a positive association with Alu-family repetitive elements have been observed, while insertions are underrepresented in LINE and LTR repetitive elements.

Overall, these results reinforce the positive risk-benefit analysis of our CAR-T cell product. Nevertheless, continued monitoring of site-integration profile in CAR-T cell products is granted. Also, understanding the molecular basis of viral vector integration mechanism and integration-site preferences is key to develop safer viral vectors for future gene therapies.

P0498

Lenti.RiGHT™ – Epigenetic targeting delivers cutting-edge lentivirus producer cell lines

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Although non-viral approaches are becoming increasingly popular, transduction with lentiviral vectors remains the dominant approach for CAR T cell generation due to its efficacy, gentleness and ease of application. Lentiviruses are also a major cost factor in CAR T therapies. They are typically produced by simultaneous transfection of four GMP-grade bacterial plasmids into HEK293 cells in suspension - a fast and flexible process with complex starting materials, cell density limitations and scale-up challenges.

Building on tight regulation and a unique, rapidly proliferating, robust HEK293 cell line that also serves as superior transient producer we have stably introduced the necessary expression units encoding envelope, gagpol polyprotein and rev into the host genome. Clonal packaging cell lines generate titres between 10^7 and 10^8 transducing units upon transfection with the lentiviral vector. However, prolonged production at high cell densities without large amounts of plasmid DNA in the preparation is only possible if all components are present in the producer cell genome. Producer cell generation is challenging and must be done separately for each CAR T product: High- producers with titers of above 5x10^7 are rare, and the time required to develop them is much longer than for a transient process.

To overcome these limitations, we combined the key elements with DirectedLuck®, a highly potent piggyback-type transposase engineered to read epigenetic marks in the host. It positions genes flanked by optimized transposon ends only in the vicinity of the most active promoters in a given cell. This not only increased the titer of bulk-pool packaging cells by a factor of 100, but also allowed the generation of highly productive producers without the need for single-cell cloning. The generation of the product-specific producer requires only a short selection and can be performed with a pre-released packaging cell line under GMP conditions. This Lenti.RiGHT® platform combines the flexibility of the transient approach with the option of intensified processes typical for the production of therapeutic proteins, leaving behind extensive plasmid production.

Process efficiency is not only determined by lentiviral titer. Transduction of T cells strongly depends on quality of the viral particles and the expression of the virus receptor. Most lentiviruses are pseudotyped with the G protein of Vesicular Stomatitis Virus, an envelope with strong fusion capability. However G protein targets the LDL Receptor that is only present on T cells after activation. We will discuss how re-targeting can improve transduction in vitro and, together with the conversion of lentiviruses to non-integrating vectors, address safety concerns for in vivo application.

P0499

Development of a dual-expression LV-based system for simultaneous transient and stable targeted gene delivery

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Lentiviral vectors (LVs) are versatile tools for genetic engineering, capable of stably transducing both dividing and non-dividing cells. LVs have been traditionally used to integrate foreign genetic material into the host's genome, enabling its stable expression. However, long-term persistence of the transgene may not always be desirable, e.g. for applications employing toxic payloads or selectors of transduced cells. To address this issue, we devised an LV-based platform that enables the simultaneous delivery of transiently and stably expressed genetic payloads to targeted cell populations.

Our engineered LVs incorporate fusion proteins which enable the packaging of an mRNA of interest into the viral particles thanks to specific aptamer-aptamer binding protein interactions. Vector production is performed by co-transfection of structural and functional LV components in 293T cells, followed by ultracentrifugation for purification and concentration. We assessed molecular and physical characteristics of the LV preps by employing ELISA and dynamic light scattering for physical particles quantification, and RT-ddPCR to assess viral RNA content. The functional evaluation of the engineered LVs was performed on various cell lines using flow cytometry or ELISA assays to detect transgene expression.

Of note, we focused on individuating strategies that enabled enhanced mRNA co-packaging into the viral particle without impacting overall LV structure and functionality, thus selecting combinations that maximized expression of both the viral genome and co-packaged mRNA. We then engineered the co-packaged mRNA to improve its half-life and expression, and included post-transcriptional regulatory sequences that enabled selective transgene expression in target cell types. Plasmid ratio in LV production were also evaluated and optimized in order to increase yield and LV prep quality. Finally, we applied our platform in different translational contexts, testing its efficiency with different co-packaged mRNA payloads and in relevant cell types.

Our system couples the benefits of mRNA transient expression with LV potential for stable transduction and favorable safety profile. This approach offers a modular, customizable tool for cell engineering that can be especially useful in applications requiring joint short and long-term transgene expression. For instance, these engineered LVs could be employed for *in vivo* vaccination strategies that co-deliver antigens with immune-activating molecules, for genome editing strategies combining temporary expression of base/gene editors with corrective DNA templates, or for the transient expression of selectors from edited cells. For these reasons, our LV-based platform represents an innovative tool that could significantly advance therapeutic and research applications in genetic engineering.

P0500

Enhancing Lentiviral Vector Production: A Scalable and Optimized Process

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Lentiviral vectors (LVs) are pivotal in gene and cell therapy, including groundbreaking immuno-oncology treatments such as CAR-T cell therapy. The growing demand for LVs necessitates the development of efficient production processes to ensure high yields and quality. This study presents the optimization of a lentivirus production process using the Ambr15® microbioreactor system for high-throughput, automated parallel cultivations. To identify key process and transfection parameters that enhance transducing titers, the MODDE® Design of Experiments (DOE) software was applied to plan and analyse the experiments.

A screening of transfection reagents at shake flask scale revealed that using FectoVIR®-LV instead of PEIpro® increased transducing titers 3-fold. In addition, DNA amounts were reduced during transfection using FectoVIR®-LV compared to PEIpro®. Further process parameter screening in the Ambr15® revealed a pH shift from 7.1 to 6.8 directly after transfection as the sole significant factor, not only improving transducing titers but also benefiting cell growth and viability. The combination of dissolved oxygen level of 30%, stirring speed of 600 rpm, and pH of 6.8 yielded transducing titers of 5.7E7 TU/mL. Additionally, a temperature shift to 33 °C was shown to enhance lentivirus titer, viable cell density, and extended the production period.

The study demonstrates a multi-faceted approach to refining the LV production process, addressing several challenges, and paving the way for more accessible LV-based cell therapies. Optimized parameters and conditions identified here offer a scalable solution to meet the increasing demand for high-quality lentiviral vectors in clinical applications.

P0501

LV integration DB, A Comprehensive Analysis of Integration Patterns of Lentiviral Vectors in Gene Therapy: Insights from the Largest Integration Site Profiling Study to Date

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Lentiviral vectors have been widely used for gene therapy and research due to their ability to integrate into the host genome and their efficient gene delivery capabilities. and are considered to offer safer integration site selection profiles and a lower degree of genotoxicity compared to γ-retroviral vectors However, several ongoing research suggests that LV integration can lead to several adverse events, including insertional mutagenesis, activation of oncogenes, and disruption of essential genes, Therefore, the safety of the integration profile, particularly in the context of potential genotoxicity and oncogenesis, is still a subject of concern. In this study, we present the

results of the largest integration site profiling to date, encompassing 91868 unique integration sites across 53 samples with lentiviral vector infection after 14 days.

This study conducted a comprehensive examination of viral vector genome insertion and its safety implications through high-throughput sequencing and bioinformatics data analysis techniques. We use LTA-PCR to enrich, amplify, and extract the DNA fragments where lentiviral integration sites are located, and then utilize Illumina Miseq for sequencing. The analysis encompassed a detailed investigation of lentiviral vector integration sites, including clonal diversity assessments, integration site preference in various functional genomic regions, proximity to cancer-associated and known adverse event-related gene, and integration hotspot analysis. These findings are instrumental in establishing a background profile of LV integration, offering a deeper understanding of the vector's genomic interaction and its implications for therapy safety and efficacy.

Integration sites of lentiviral vectors demonstrate a marked preference for gene-rich regions and regions near transcription start sites (TSS). it also shows a significant integration preference towards chromosome 11, 12, 17, and 19, which is consistent with the fact that chromosome 19 has the highest gene density among all human chromosomes. Furthermore, our analysis revealed that integration sites occurring within genes, notably those associated with oncogenic functions, tend to exhibit a substantially higher clonal prevalence ($p<0.01$), this implies that integration into a specific site could provide an evolutionary advantage to the cell. In prior clinical investigations, integrations associated with adverse events have been identified within the STAT5B and BACH2 gene. Analysis of our dataset has uncovered a marked increase in the frequency of lentiviral vector-mediated integrations at the above 2 genes ($p<0.01$). Nonetheless, the proportion of clones harboring these integrations remains minimal (with a median of 0.008%). This indicates that integration events in AE associated genes still warrant attention and monitoring, suggesting a potential impact on clonality in gene therapy applications.

Our study stands out as the most extensive investigation of LV integration sites thus far, providing a public available dataset for future research, offering valuable information that can enhance our understanding of LV integration mechanisms and guide the development of safer and more effective gene therapy strategies.

P0502

Overexpressing an engineered soluble TβRII controlled by an NF-κB-Inducible promoter to reduce fibrosis after skeletal muscle injury

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One of the main challenges in regenerative medicine is achieving successful regeneration after extensive musculoskeletal injuries, which are characterized by a persistent inflammatory environment accompanied by the release of the fibrotic trigger TGF-β1. This leads to unhealthy production of scar tissue, including reduced expression of myogenic genes and enhanced expression of fibrotic and atrophic genes. Thus, we hypothesize that overexpressing the soluble receptor of TGF-β1 (sTβRII) to sequester excessive TGF-β1 released after skeletal muscle injuries could reduce fibrosis induced by TGF-β1-TβRII binding *in vivo*. Due to its sustained expression, low toxicity and immune response, and high transduction capacity, two lentiviral vectors were

designed and produced: Lv_NF-κB_sTβRII and Lv_NF-κB_Luci (control). Therefore, for *in vitro* studies, cultured C2C12 cells were transduced, and injured with $BaCl_2$, with gene expression evaluated by RT-qPCR. For the *in vivo* tests, mice were divided into control (n=5), injury (n=5), and injury with treatment groups (n=5). They were injured in the anterior tibialis muscle by contusion, and Lv_NF-κB_sTβRII was injected into the site of the lesion. Functional analysis was performed on a treadmill and on a ladder. Molecular analysis was conducted by plasma TGF-β1 ELISA and by RT-qPCR to assess inflammation, fibrosis, myogenic differentiation, and expression of the soluble TGF-β1 receptor. For statistical analysis, all of these were conducted using GraphPad Prism 9.0 software with a 95% confidence interval. In the *in vitro* results, RT-qPCR showed a 442-fold increase in the number of sTβRII copies at 24h and a 67-fold increase at 48h in C2C12_NF-κB_sTβRII compared to the control, revealing inflammation-induced expression. The injury/treatment group displayed superior treadmill performance compared to all other groups, matching the control group's maximum load (36g), with 40% of the animals adhering. This represented a decrease of 20% in the frequency of animals compared to the control group, which had a frequency of 60%. Conversely, the injury/treatment group still exhibited the shortest average climbing time among all groups. In contrast, the injury group showed the poorest treadmill performance over 30 days compared to all other groups, with the lowest average support on the ladder. Additionally, only 20% of the animals in the injury group could support the group's maximum load (31g) which had the longest average climbing time compared to all other groups. Plasma ELISA of TGF-β1 indicated no significant difference in systemic TGF-β1 at 7, 16, and 34 days, suggesting localized transgene expression. RT-qPCR (n=5) showed significantly higher expression of the inflammation gene *NF-κB*, the anti-inflammatory gene *IL-6*, the anti-fibrotic marker *Smad7*, the transgene *sTβRII*, and the myogenic differentiation markers *Pax7* and *MyoG* in the injury/treatment group compared to all other groups. *Myod* expression was significantly higher in the injury/treatment group compared to the injury group.In conclusion, it was observed modulation of *sTβRII* expression via the *NF-κB* inducible promoter and enhanced muscle regeneration through TGF-β1 sequestration. Moreover, the Lv_NF-κB_sTβRII vector improved muscle function, performance, and increased expression of myogenic differentiation markers in mice.

P0503

Retrieval and Quantification of Vector Integration Sites by Sonication Linker Mediated (SLiM)-PCR: Optimization Process

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In gene therapy (GT) applications based on integrative vectors, the retrieval and mapping of vector integration sites (IS) in the genome of transduced cells and their progeny allows to obtain safety and efficacy readouts of the treatment. This is achieved using specialized PCR-based techniques combined with next-generation sequencing (NGS) and bioinformatics analyses

We have developed a method for retrieving IS called Sonication Linker Mediated-PCR (SLiM-PCR), which relies on specific PCR amplification of vector/genome junctions from sheared genomic DNA followed by NGS. The identification and quantification of IS are performed using dedicated software, VISPA2 and ISAnalytics. The abundance of each IS is determined by counting

the number of amplified DNA fragments of varying lengths (dependent on the shear site position) containing the same IS, which correlates with the number of cells contributing to a specific clone, thus avoiding PCR amplification biases.

SLiM-PCR has demonstrated high efficiency in retrieving IS and accurately quantifying a single lentiviral (LV) or γ-retroviral (γ-RV) IS down to 0.1% relative abundance. This accuracy was demonstrated in an experimental setup where SLiM-PCR was applied to DNA standards composed of monoclonal samples carrying a known number of IS at specific genome positions, mixed with a polyclonal sample harboring a random IS distribution.

Due to its accuracy and efficiency, we have adopted SLiM-PCR as the reference assay for IS analysis. SLiM-PCR is currently being applied to thousands of samples from over 30 preclinical studies and 7 different GT clinical trials utilizing various vector types, including γ-RV, LV, Sleeping Beauty Transposons, and Adeno-Associated Vectors.

SLiM-PCR has been fully automated with liquid handlers capable of processing hundreds of DNA samples per week in 96/384-well plates. We are working on miniaturizing the reactions using the Acoustic Droplet Ejection technology of the Beckman Labcyte 525, which can move nanoliters of liquids using acoustic waves. This has allowed us to reduce reaction volumes tenfold, down to 5 microliters, achieving cost reduction, increased sample throughput, and improved overall assay speed and reproducibility. We tested the miniaturized SLiM-PCR on DNA standards using varying amounts of genomic material and vector copy numbers (VCN), PCR amplification conditions, and reagents to find the optimal balance between miniaturization and performance. Miniaturized SLiM-PCR produced comparable or superior results to standard volumes in terms of the number of IS retrieved from the same amount of genomic DNA, maintaining precision in IS abundance estimation and detection limit.

To decrease false positives and sequence-sample misassignments caused by index hopping, we are comparing the emerging MGI DNBseq technology to the Illumina NGS technology. We are investigating if the potentially lower error rate provided by rolling circle amplification, as opposed to conventional exponential amplification during sequencing, might offer benefits.

Overall, our findings indicate that SLiM-PCR is a reproducible, accurate, and versatile method for high-throughput IS analyses, with potential for further improvement through ongoing optimization efforts.

P0504

Short- and Long-read Sequencing for Integration Site Analysis (ISA) of Viral Vectors for Gene Therapy

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While lentivirus has historically been the viral vector of choice for gene therapy, recombinant adeno-associated viral (rAAV) vectors have seen widespread application as a gene therapy delivery platform in preclinical and clinical studies. Unlike lentiviral vectors, which are fundamentally integrated into the host genome, recombinant AAV largely remains episomal after transduction into the nucleus of the host cells; however, data has shown AAV may integrate into the host genome in a random, non-homologous manner. The frequency of AAV integration has been estimated at 0.1% to 10% in hepatocytes. In consideration of the theoretical risk of

tumorigenesis associated with lentiviral and AAV integration in humans, assessment of viral vector integration in *in vitro* studies, animal models, and patient samples are recommended by FDA for preclinical and clinical studies and long-term follow-up studies (LTFU). AAV integration analysis presents several challenges, including the random nature of AAV integration sites and low expected integration frequency. To address this, Next Generation Sequencing (NGS) for viral vector integration analysis has been adopted as a highly sensitive assay. Traditional approaches utilize targeted enrichment sequencing with hybrid-capture or linear amplification PCR in combination with short-read sequencing technology. The addition of long-read sequencing offers the advantage of being able to identify chimeric reads with clear sequence structure as genome-insert-genome. Here we describe multiple mixed ISA approaches in lentiviral and AAV studies, with improved sensitivity and specificity for ideal detection of viral vector integration.

P0505

XOFLX™ stable cell lines: a flexible platform for lentiviral vector production

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We have previously developed stable lentiviral vector (LVV) packaging and producer cell lines. Typically, these cell lines yielded 1.5 ×10^8 TU/mL with EGFP as the cargo gene, and tests with therapeutic cargo genes yielded consistently high titres across platforms. Moreover, these cell lines were stable over 27 passages, both in terms of LVV infectious titre and copy number of integrated genes. LVV from producer cell line tested negative for replication competent lentiviruses (RCL). The aim of the work presented here was to further test the performance of our stable cell lines with cargo gene expression driven by the EF-1α and EFS promoters, which are commonly used in LVV products. Since these are of human origin, it is expected that they would be less readily silenced than would viral promoters by production cells and target cells. LVV titres with different promoters and different cargo genes (anti-CD19 CAR and anti-BCMA CAR) were consistent across platforms. In addition, we investigated means by which to expedite development of custom packaging and producer cell lines. Our approaches included: simultaneous piggyBac transposase-mediated integration of LVV elements encoded in multiple constructs; single cell isolation shortly after plasmid integration; and methods to improve the reliability of clonal cell line expansion by limiting the negative effects of cell line isolation. We have shortened the development time by six weeks through optimisation of our cell line development methods. Finally, we present developments on our cargo gene silencing system. We engineered a method to selectively silence the cargo gene mRNA in vector-producing cells and, together with the insertion of a universal target site outside the cargo gene CDS, we have achieved up to 95 % cargo gene silencing with a universal shRNA. This cargo gene silencing system is anticipated to improve stable cell line stability and improve vector quality when encoding challenging cargo genes. The developments presented here further demonstrate the robustness and flexibility of the XOFLX™ platform in facilitating the development of urgently needed cell and gene therapies.

P0506

Lentiviral vector manufacture ad-hoc optimization and development strategies to overcome cell-therapy cost-effectiveness and regulatory hurdles: a real-life example

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VIVEbiotech is a biotech (CDMO) fully specialized in Lentivirus-based transfer technologies. Our mission consists in providing LVV from very early stages through our innovation department to GMP for clinical and commercial in a robust and cost-effective manner. To accomplish it, one of our high values is the support we give from the very early-development phases. Our particular focus on LVV project optimization in collaboration with our partners supported by our long clinical development field experience, offers solutions in a timely and cost-effective manner. This allows reducing the cost of therapies per patient by enhancing the productivity and functionality of our partners' lentiviral vectors before transferring them to our highly scalable manufacture platform.

In this presentation, we will report an optimization case study performed in collaboration between VIVEBiotech's Innovation Department and Advesya, a cell-therapy company that employs our LVV to engineer their CAR-T product, that illustrates how developers and CDMO can bring together successful LVV manufacture improvement strategy. In this specific case, two main optimization strategies were followed with a powerful impact on the whole cell-therapy manufacture process.

Firstly, optimization of lentiviral vector construct by removing non-relevant sequences for CAR-T function improved viral genome packaging efficiency that translated into a 2.5-fold increase in LVV productivity. Relevantly, construct modulation boosted by 2-fold CAR expression capacity on T-cells bringing the possibility of reducing required viral quantity for the subsequent CAR-T manufacture process.

Secondly, optimization of transfection step of LVV manufacture process in terms of packaging plasmid configuration and the ratio used for LVV manufacture allowed for a significant drop of manufacture-process related contaminants content and an additional improvement of LVV productivity reaching up to 3-fold productivity improvement achieved by construct optimization.

The synergic effect of the followed optimization approaches brought relevant improvements, not only on LVV production capacity but also on vector efficacy for CAR-T generation, that translated into a 7-fold improvement of the whole therapy cost-effectiveness with a relevant improvement of vector quality.

VIVEbiotech can therefore provide a fully integrated optimization and large-scale LVV manufacture services pursuing partners' project improvements from early stages reaching for a powerful impact on therapy cost-effectiveness and quality that ensure transferability of partner's projects to clinical and commercial phases.

P0507

Cost reduction of lentivirus manufacturing for CAR-T by transfection optimisation to minimise the required quantity of plasmid DNA using Design of Experiments

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Lentiviruses (LV) are commonly used to introduce Chimeric Antigen Receptor (CAR) transgenes into T cells for CAR-T therapy. High costs associated with large-scale LV manufacturing are a significant obstacle for the development of CAR-T therapies. Improving the cost-effectiveness of the LV manufacturing process is key for accelerating clinical development of CAR-T therapies. Transfection is a critical step in typical lentivirus manufacturing processes, during which plasmid DNA (pDNA) is introduced into producer cells, enabling the producer cells to produce LV. pDNA and transfection reagents (TR) such as polyethylenimine (PEI) are costly at GMP-grade, making the transfection step a significant part of the Cost of Goods (COG) of the LV manufacturing process. Optimising the transfection step to reduce the amount of pDNA that is needed can significantly reduce the cost of the manufacturing process. In this study, the effect of various process parameters on LV functional titre and cost was investigated using Design of Experiments (DOE), a methodology that allows the studying of the effect of multiple factors and their interactions on a response variable. Initially, a response surface design was created and executed at small scale to find the optimal setpoints for viable cell density (VCD), pDNA quantity and transfection reagent quantity. Secondly, an optimal design was created and performed at small scale to optimise the plasmid ratios and pDNA quantity. Finally, the optimised setpoints were used to demonstrate scale-up potential by producing LV at 1.5 litre scale in a Wave bioreactor. The optimised factor values resulted in a reduction in pDNA quantity and PEI quantity to less than half of the initial, literature-based values, significantly reducing COG while maintaining high functional LV titres. In addition, the statistical models created with the experimental data gave valuable insights into the process. This improved process understanding could be used as the base for an appropriate control strategy for the transfection step, and gives insight into how to further improve the process. These results show that using a low cell density, low pDNA transfection process is a viable approach to reduce the cost of LV manufacturing without compromising on titre. Using these low-cost processes can aid in making LV-based therapies more affordable and accessible.

P0508

Maximising lentiviral vector yield while minimizing impurities with a robust and scalable purification platform utilizing an innovative nanofiber absorbent

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Lentiviral vectors (LVVs) are essential for long-term and stable gene expression in both dividing and non-dividing cells. However, their high sensitivity to factors such as low pH, high salt,

temperature changes, and shear forces makes them difficult to process. The significant presence of host cell proteins (HCPs) in suspension LV feedstock further complicates LVV purification. To address these issues, we developed a next generation chromatography matrix that allows LVV purification under mild conditions, such as low salt elution and low shear stress environment, together with high binding capacity, that can be operated at speed under low operational pressure. This study aims to optimize the downstream processing workflow to maximize LVV recovery and purity when using this novel nanofiber adsorbent. The objectives of this study are: 1) To optimize the clarification step prior to LVV capture at the bench-scale, using the nanofiber adsorbent in a spin column format as a rapid screening tool; 2) To establish a lentiviral purification workflow from lab-scale to process development scale, that maximizes lentiviral recovery and impurity removal, using the nanofiber adsorbent in a capsule format compatible with chromatography systems; 3) To test various clarification methods on suspension LVV feed prior to lentiviral capture and assess LVV recovery and impurity removal, including residual dsDNA and HCPs. In conclusion, regardless of the clarification methods tested, this purification platform consistently achieved robust recovery yields of functional LVVs, with high clearance of HCP and dsDNA.

P0509

Automated LVV titration assay to increase laboratory capacity

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AGC Biologics is a global CDMO providing development and manufacturing services for protein-based biologics and advanced therapies, including viral vectors and genetically engineered cells.

Lentiviral vectors (LVV) are vehicles for efficient gene delivery that play an important role in ATMPs. The efficiency of the production process and the performance of the final LVV is monitored through functionality and purity methods.

At AGC Biologics, the determination of the infectious viral titre is based on the quantification of the proviral DNA copy number integrated into the cell genome of a reference cell line that is well characterised and fully representative of target primary cells. Cells are transduced with serial dilutions of vectors and tested with a qPCR assay. To address the significant bottleneck posed by the large number of samples to be tested, especially during process characterisation, various steps of the entire assay have been automated using Hamilton-based systems. This includes automating LVV serial dilution during cell transduction, pellet preparation, and the full setup of the qPCR assay. The goal is to achieve a completely automated flow from sample dilution to the final result.

Hamilton's automated solutions have been designed and optimised using a scheduler that enables the parallelisation of some of the steps of the automated method with the aim to reach a 5-fold increase in the number of samples tested per day, thus significantly reducing analysis time and operator effort. The automated system consist of a core Hamilton liquid handler that, in the case of qPCR assays, is fully integrated with all the devices needed to perform the different steps, including dilutions of standards, controls and samples, reagents preparation, plate loading, assay run and raw data generation.

The automated method for LVV titration, qualified according to ICH Q2 (R1), demonstrates repeatability of ≤ 15% and inter-assay variability between automated and manual execution of ≤ 19%. Its performance is fully aligned with the manual method, showing comparable results on samples from various purification steps of the LVV production process and final purified LVV. These automated solutions designed to increase the number of processed samples allowed us to reach more than 1000 tested samples per year for the characterization of 61 batches, with an average of more than 80 samples per month. The CFR21-compliant automated system allows to have a complete automatic and traceable flow from sample vial to final result, decrease the variability associated to the operator execution, and shorten the timelines that makes the difference in laboratory capacity.

P0510

A novel lentiviral production system to minimise transgene expression in producer cells

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During the manufacture of lentiviral (LV)-based therapies using HEK293 transfection methods, there are often undesirable levels of transgene expression in producer cells. This expression may be expected where the internal transgene promoter is constitutively active. However, in HEK293 producer cells where this internal promoter should be silent, high levels of transgene expression are often still observed. This is largely driven by the promoter upstream of the 5' LTR that is responsible for transcription of the RNA which ultimately gets packaged into mature LV particles. This so-called breakthrough expression may negatively impact titres, particularly if the transgene encodes a protein which is toxic to producer cells or interferes with vector assembly. Further, it has been previously shown that if these proteins are incorporated into the LV particle surface, it may result in the unintentional transduction of certain cell types. A treatment utilising an anti-CD19 CAR vector preferentially targeted malignant B-cells in a B-cell acute lymphoblastic leukaemia (B-ALL) patient, rendering it ineffective due to reduced binding of the vector to T-cells.

To address these challenges, we have engineered a novel promoter, upstream of the 5' LTR, which drives transcription of the viral genome and results in the production of infectious LV particles. A number of promoter sequences were tested, each incorporating different regulatory regions, and utilising a GFP transgene to assess breakthrough expression and resulting titres by flow cytometry. This novel viral genome promoter was compared to industry standard CMV in a 3rd generation backbone, with the transgene under the control of a liver-specific LP1 promoter, resulting in up to a 100-fold decrease in breakthrough expression in producer cells. Where the transgene is under the control of a constitutively active promoter in HEK cells, such as GAPDH, a 10-fold decrease is observed.

When tested with clinically relevant payloads including FVII, COX2 and LDLR, similar decreases in transgene expression were observed, whilst producing functional LV particles. A further production of an anti-CD19 CAR vector, which was concentrated by centrifugation, resulted in a higher functional titre using this novel promoter design of 1.8E8 TU/ml, compared to 1.2E8 TU/ml in a regular 3rd generation backbone, determined by qPCR. This may be due to expression of the transgene compromising producer cell fitness, and therefore production.

These data demonstrate that this novel promoter is able to significantly reduce transgene expression in producer cells, whilst still resulting in the production of functional LV particles. We propose that observed reduction of transgene expression may be attributed to splicing being modulated, since the only difference in designs is the promoter upstream of the viral 5' LTR. Overall, this presents a possible LV platform to enhance the quality and potentially quantity of LV production.

P0511

Lentiviral vector encapsulation by alginate hydrogels for enhanced delivery in a 3D skin model

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Recessive dystrophic epidermolysis bullosa (RDEB) is a severe genetic disorder caused by mutations in the COL7A1 gene, leading to chronic skin fragility, blistering, and fibrosis. This condition significantly impacts patients' quality of life and currently has no cure.

This study aims to develop a new treatment platform for skin diseases, combining lentiviruses (LV) and biomaterials in a 3D skin model, to create a foundational tool for potential gene therapy applications for RDEB.

LV are effective gene therapy vectors due to their ability to transduce both dividing and non-dividing cells. Engineered to be replication-incompetent, they ensure safety and their large cargo capacity enables the delivery of complex genetic material. Additionally, they are less prone to immune responses, enhancing their stability and persistence in the host.

Alginate hydrogel is a biocompatible biomaterial used in drug delivery and tissue engineering. One of its primary benefits is its ability to form hydrogels, which can encapsulate sensitive biological agents like viral vectors without causing damage, while also having the capacity to be modified for controlled release. Its porous structure supports nutrient and oxygen diffusion, making it ideal for 3D cultures and tissue models.

To enhance transduction, low molecular weight 1% oxidized alginate hydrogels were used to encapsulate LV (carrying the GFP gene), providing controlled release and protection from degradation, improving stability and delivery. The LV-alginate hydrogel system was tested in a 3D skin model, to simulate in vivo conditions, in four conditions: negative control (only skin), positive controls (skin only with alginate hydrogel and skin only with virus), and the test group (skin with LV-alginate system). Each condition was tested in duplicate, and the skin was collected and processed after 72 hours.

After the test, immunofluorescence confirmed GFP transgene presence, indicating transduction efficiency. ImageJ program was used to quantify the presence of green cells and for the result we utilized Welch's ANOVA due to unequal variances across groups (Levene's test, $p < 0.001$), which revealed significant differences between the groups ($p = 0.020$). Subsequent Games-Howell post-hoc analysis showed that the mean difference in GFP expression between the control groups and the virus encapsulated in alginate hydrogel was substantial, indicating higher transduction efficiency with the alginate encapsulation. These results suggest that alginate hydrogel

encapsulation significantly enhances the efficiency of lentiviral transduction in 3D skin models, increasing it up to five times compared to the virus only group.

Finally, to test the gel toxicity, an ELISA was performed to detect the presence of IL-6 in the supernatant of the 3D skin. No difference in IL-6 concentration was detected, indicating that the alginate and the virus are not inducing immune responses.

In this study we developed a system using LVs encapsulated in biomaterials to enhance gene delivery in 3D skin models, offering a promising platform for future research in gene therapy. Here we explored the synergy of lentiviral vectors with biomaterial scaffolds, potentially paving the way for new therapeutic strategies for genetic skin disorders like RDEB.

P0512

A retargeted envelope for in vivo CD4+ and CD8+ T-cell targeting with reduced liver off target

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Advances in T cell engineering have introduced promising therapeutic options. Genetic modification of autologous T cells has successfully treated cancer patients and shows potential for autoimmunity and age reversal. Traditionally, T cells are modified ex vivo, posing significant cost and scalability challenges. In contrast, in vivo modification could streamline this process. Various viral systems have been explored to enhance T cell targeting. For instance, a binding-deficient VSV-G variant, combined with cell-specific targeting molecules, has shown potential for in vivo CAR-T targeting. We have developed FUSOBIN, a new engineered pseudotype have enabled precise targeting of CD4+ and CD8+ T cells. This system has a reduced liver off-target and high immune invisibility. We will describe FUSOBIN molecular principles and show potencial for in vivo T cell engineering.

P0513

Precision Mapping Viral Integration Landscapes with CRISPR-Cas9 and Long-Read Sequencing

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Gene therapies utilize both integration and non-integrating viral vectors. Integrating vectors such as lentiviral (LVV) and retroviral (RVV) vectors, are used for stable expression by integrating a therapeutic gene into the host's genome. The LVV or RVV are used in cell and gene therapies (CGTs), especially for CAR / TCR-T (Chimeric Antigen Receptor / T-Cell Receptor) therapies. LVV/RVV vectors follow random integration patterns and concerns regarding vector genotoxicity by activation of proto-oncogenes, warrant safety testing. Vector integration sites (VIS) in the host genome needs to be precisely identified and reported, to confirm that the therapeutic gene is safely inserted into the genome. We developed an unbiased targeted approach using CRISPR-

Cas9 for enrichment followed by sequencing on Oxford Nanopore's Mk1C sequencer to identify VIS in WHO (World Health Organization) control material. The WHO control sample has VIS reported at 10 different genomic locations. We designed guide RNAs that target conserved regions in the lentiviral vectors to selectively enrich for regions of interest in a background of human DNA. After enrichment, sequencing libraries were made for Oxford Nanopore long-read sequencing, using the latest V14 chemistry. The power of long read sequencing is utilized here in such a way that part of the viral vector is sequenced together with adjacent part of the human genome, thereby precisely mapping the integration sites. The sequencing runs met all quality control criteria and no errors occurred. Basecalling was done using the latest software tool developed by Oxford Nanopore Technologies. The resulting data were analyzed using a custom-made bioinformatics pipeline, which essentially identifies integration sites by mapping reads to both the viral vector and human reference genomes, determining the alignment breakpoints of the chimeric reads. Interestingly, our results indicated that by altering the gRNA binding in *cis* or *trans*, we could vary the depth of sequencing for the viral vector. Using a combination of the gRNAs and long read sequencing, we could enrich and reliably detect all the ten reported integrated sites in the WHO control sample. Our pipeline enables sensitive and reliable detection of integrated LVVs used in cell and gene therapies (CAR / TCR-T and other gene therapies). In addition to VIS, it can identify potential recombination, if any, of the vector that might cause replication competency or mutations that hinder the therapeutic gene expression. We are exploring our VIS pipeline for other viral vectors including RVV and AAV vectors.

P0514

Design of experiments for lentiviral downstream process optimisation on CIM QA 0.05 mL Monolithic 96-well Plates

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Lentiviruses are being increasingly used as tools for gene delivery. Currently, their primary use is *ex-vivo*, however, *in-vivo* applications will soon follow, for which sufficient purity and infectivity, will be required. To address these issues, monolithic columns have been tested for downstream purification, with good initial results on CIMmultus QA. To further improve the downstream process, we decided to determine the factors and conditions, which contribute the most to downstream recovery, by using Design of Experiments (DoE). To increase throughput of the DoE studies, we used CIM QA 0.05 mL Monolithic 96-well Plates, which significantly sped up the testing process, as well as decreased variables which occur from day-to-day work. From the factors tested in DoE, results indicate that starting salt concentration in lentiviral load has the most profound effect on lentiviral recovery. With the DoE help, we could improve the infective lentiviral recovery from 20% to over 60%. Improved understanding of conditions important for lentiviral downstream will ease future process development. With additional process improvements, monoliths have great potential in enabling scalable and cost-effective downstream process solutions for lentiviruses.

P0515

Automated solutions for characterization and release of DP

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In the field of gene therapy, an efficient delivery of the therapeutic gene and a proper gene expression are crucial to obtain clinically relevant effects. Lentiviral vectors (LVV) are effective transfer system for the introduction of transgenes into target human primary cells, such as CD34+ cells. AGC Biologics has developed and optimized a manufacturing process for LVV transduction of primary cell lines. This process includes a comprehensive set of in-house developed tests for the characterization of the Drug Substance (DS) and the release of the final Drug Product (DP).

For the release of the DP, two methods are in place at AGC Biologics to assess the efficacy of the transfer of the vector into the final target cells: the quantification of the mean number of the provirus integrated into the host genome (VCN) and the transduction efficiency (TE) on CD34+ derived colonies. Both methods use a Real Time PCR assay and have been automated with the aim of increasing throughput, shortening timelines and decreasing operator effort.

The Hamilton's automated liquid handler for qPCR execution is integrated with a variety of devices, including a Real Time qPCR thermal cycler and a plate sealer. This integrated system allows the continuous process of samples without operator attendance, thus performing a complete automatic flow from reagents and sample preparation to run execution and obtaining of raw data. While for VCN determination, the lysate of transduced cells is directly processed by qPCR, the TE includes the harvest and lysis of colonies derived from transduced CD34+ cells, previously seeded on a methylcellulose substrate. A fixed number of colonies are tested and the transduction efficiency is determined as the percentage of transduced colonies.

The methods were validated before automation, and the automated performance has been aligned with the manual methods for each assay. This alignment was demonstrated with a repeatability of ≤ 14% and inter-assay variability of ≤ 19% compared to the manual execution.

Moreover, different ELISA assays are executed at AGC Biologics for the verification of the removal of impurities during the transduction process (e.g. cytokines or Host Cell Proteins -HCP- deriving from reagents added during cell culture or coming from vector). Among them, the ELISA for 293T HCP has already been transferred to automation. In this case, the automated system has been conceived with a different requirement, to combine the processivity with the flexibility to further add other assays to be run together to share the same sample aliquot. This Hamilton automated system is completely integrated with all devices needed to perform each step of ELISA assay, including a washer, incubators and a reader, allowing a complete traceable flow of the tested samples up to the final result. The automated method assures a comparable performance with the manual method ($p \geq 0.26$) and has been qualified according to ICH Q2(R1) with both repeatability and intermediate precision ≤ 20%.

The CFR21-compliant automated system, allows reaching a 2-fold increase in throughput, shortening the timelines and making the difference in laboratory capacity and during process characterization or DP release.

P0516

Rational design affinity convective chromatography to streamline lentivirus purification

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The existing lentiviral vector purification process takes long processing time and suffers low purification efficiency. Therefore, if use affinity chromatography the lentivirus will have a higher concentration and purity, which can effectively reduce the process steps and save production costs. Lentivirus is labile, we can't use the nanobody scaffold instead we retional design peptide ligand by using peptide docking tools. Meanwhile lentivirus is large (80-100nm), how to design conjugate the scaffold to stationary is crucial, which affects the performance. The author developed double ACC (Affinity Convective Chromatography). Firstly, the impurities such as free VSV-G,HCD and HCP were removed by Phospholipid Affinity. VSV-G pseudoaffnity is then used to further remove other protein impurites and ineffective virus particles such as exosomes that do not contain VSV-G proteins. The lentiviral vector with higher purity, higher effective viral particle number ration (TP/IP), lower impurity content and safer can be obtained by two-step affinity superposition. Both stages of affinity can be eluted under mild conditions to ensure maximum viral activity.

P0517

Advanced nuclease applications in Lentiviral vector bioprocessing for superior downstream recovery and vector product quality

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Efficient and robust downstream processing of Lentiviral vectors (LV) is critical for producing high-quality gene therapy vectors. Traditional nucleases used in LV manufacturing often result in sub-optimal vector recovery and high residual DNA levels in the final drug product.

This project aimed to identify and integrate alternative nucleases, namely Salt Active Nuclease and Medium-Salt Active Nuclease, into Oxford Biomedica's LV manufacturing workflows to enhance vector recovery and improve overall product quality. Key characteristics of alternative nucleases such as optimal pH and salt buffer conditions were evaluated and incorporated into downstream processes and compared to traditional nuclease-based downstream processes.

Our findings demonstrate that the use of Salt Active Nuclease and Medium-Salt Active Nuclease exhibited superior activity under typical LV manufacturing conditions. Notably, the incorporation of alternative nucleases reduced vector aggregation during purification and improved around two-fold vector recovery during the challenging sterile filtration step of Vector Product processing. Most importantly, the incorporation of these nucleases resulted in markedly lower levels of residual DNA in the final drug product, addressing a critical quality attribute for gene therapy applications.

LVs offer significant promise for effective cell and gene therapy applications, and the adoption of new therapies will require efficient methodologies for their production and purification. This is particularly needful when considering use of LVs in an *in vivo* setting. The incorporation of alternative nucleases into the manufacturing process presents a viable strategy for the effective residual DNA removal and improved downstream processing. These advancements hold promise for enhancing the efficacy and safety of LV-based cell and gene therapies.

P0518

Establishment and Characterization of a Stable Producer Cell Line Generation Platform for the Manufacturing of Clinical-Grade Lentiviral Vectors

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To date, nearly 300 lentiviral-based gene therapy clinical trials have been conducted, with eight therapies receiving regulatory approval for commercialization. These advances, along with the increased number of advanced-phase clinical trials, have prompted contract development and manufacturing organizations (CDMOs) to develop innovative strategies to address the growing demand for large-scale batches of lentiviral vectors (LVVs). Consequently, manufacturers have focused on optimizing processes under good manufacturing practices (GMPs) to improve cost-efficiency, increase process robustness, and ensure regulatory compliance. Nowadays, the LVV production process mainly relies on the transient transfection of four plasmids encoding for the lentiviral helper genes and the transgene. While this method is efficient at small scales and has also proven to be scalable, the industry is exploring alternative processes due to the high cost of GMP reagents, and the batch-to-batch variability predominantly attributed to the transfection step. Here, we report the development and implementation of a reliable and clinical-grade envisioned platform based on the generation of stable producer cell lines (SCLs) from an initial well-characterized lentiviral packaging cell line (PCL). This platform enables the production of VSV-G-pseudotyped LVVs through a fully transfection-free manufacturing process. Our data demonstrate that the developed platform will facilitate successful technological transfer to large-scale LVV production for clinical application. With this simple and robust stable cell line generation strategy, we address key concerns associated with the costs and reproducibility of current manufacturing processes.

P0519

Process development to enhance cost-saving benefits of XOFLX™ Packaging and Producer Cell Lines for lentiviral vector manufacturing

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We have developed XOFLX™ lentiviral packaging and producer cell lines to reduce or eliminate plasmid transfection during lentiviral vector (LVV) manufacturing, thus saving plasmid manufacturing cost, simplifying supply chain and improving process robustness of LVV manufacturing. Flask-scale production testing with various therapeutic cargo genes – including anti-CD19 CAR, anti-BCMA CAR and Factor VIII – revealed consistency in the performance of the packaging and producer cell lines compared to the industry-standard four-plasmid transfection method. To further enhance the cost-saving benefit from XOFLX™ cell lines, we've carried out various process development work to increase LVV productivity in the packaging and producer cell lines.

Our standard process in shake flask format uses Medium A for both cell maintenance and LVV production. While scaling up the production to stirred tank reactors (STR), we also tested Medium B and the corresponding Feed B to try to increase the upstream titres of LVV production from XOFLX™ packaging cell line. The optimal concentrations of Medium B and Feed B managed to increase LVV titres by 60-100% in shake flasks and 2L STR. Further scaling up to 10L STR using the optimal condition resulted in 1.4E8 TU/mL infectious titre in clarified LVV samples. Meanwhile, we tested various pH settings during LVV production in STRs, and found that reducing pH 24 hours post transfection improved LVV infectious titre by ~3 fold.

XOFLX™ producer cell lines, on the other hand, have even greater potential in LVV titre improvements. They not only can be easily scaled up from shake flasks to various scales of STRs without much need of process optimisation, but are also compatible with continuous harvesting process, which can result in much higher LVV yield in each production run. To establish the continuous harvesting process, we firstly tested cell outgrowth and multiple harvests in a scale-down model in shake flasks. Without adding the inducer, by refreshing culture media regularly, we managed to grow the producer cells to high density (>9E6 cells/mL) with good viability (>95%), showing the potential of intensifying the LVV production process. By adding the inducer and keeping the media exchange rate, we managed to do multiple harvests in an extended harvesting window, and the total LVV yield was increased by >5 fold in this intensified process compared to the standard process. Next step, we'll apply the high cell density and media exchange regime to KrosFlo® TFDF® system to establish the proper continuous harvesting process to release further potential of XOFLX™ producer cell lines.

By developing the LVV processes of XOFLX™ packaging and producer cell lines, we managed to significantly improve the LVV titres from both platforms, enhancing the cost-saving benefits and commercial viability of the platforms.

P0520

Towards a directed evolution approach to improve lentiviral vector targeting

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The use of recombinant lentivirus (rLV) holds significant promise for treating a variety of monogenic disorders due to the vector's capacity to transduce a broad spectrum of cell types, including non-dividing cells. Despite this potential, the efficiency and specificity of rLV targeting remain limited, needing strategies to minimize off-target effects and reduce dependency on *ex vivo* gene delivery methods. Here, we develop methods necessary to allow directed evolution

approaches to select novel pseudotypes to enhance the cell-type specificity of rLV vectors which incorporate the F and HN glycoproteins from *Sendai virus strain Z*. These F and HN glycoproteins have been used to generate the F/HN pseudotype to target the airway epithelia. Our methodology employs a double-round viral production strategy using a vector genome backbone with intact U3 regions that can conditionally (via provision of Tat) produce viral RNA (vRNA), enabling vector mobilisation. As a proof-of-concept, VSV-G pseudotyped mobilisable rLVs encoding either F, HN or an EGFP transgene under the control of a tetR-sensitive transgene promoter, were produced by transient transfection of HEK293T-tetR (NTR1) cells. These vectors had functional titres of 7.1±2.0e8, 6.5±1.7e8 and 3.0±0.6e8 TU/mL for the F, HN and EGFP variants respectively. In a second round of viral production, these VSV-G pseudotyped particles were used to transduce NTR1 cells. Subsequently, transgene expression (F or HN) was induced with doxycycline and the remaining components necessary for viral production (GagPol, Rev, Tat) were introduced via transient transfection, as well as either HN for the F encoding vector or F for the HN encoding vector. This sequence of events enabled the formation of rLV particles which were self-pseudotyped (i.e. the vector genome in any given vector particle encoded, as appropriate, the F or HN pseudotyping protein found on the particle surface). In the first instance, functional titres achieved were modest - 4.9e4 and 12.4e4 TU/mL for F and HN self-pseudotypes respectively. However, conventional manufacturing scale-up and vector concentration approaches will allow the creation of bar-coded libraries of F and HN sequence variants created by error prone PCR and/or closely related sequence shuffling with sufficient diversity to allow selection of novel pseudotypes via directed evolution. We anticipate this process will allow for the refinement of F and HN glycoprotein variants with enhanced cell-type specificity and improved transduction efficiency.

P0521

Non-integrating lentiviral vectors in the delivery of Cas9 nickase and homologous template for targeted transgene integration

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Genome editing strategies such as base editing and prime editing have been developed to enable safer and more efficient small modifications to the genome. However, in some gene therapy applications the delivery of an entire transgene is still required. In these cases, lentiviral vectors (LVs) are a good option for transgene delivery: they can transduce both dividing and nondividing cells, and have a wide tissue tropism. Furthermore, the packaging capacity of LVs is much larger than that of adeno-associated virus vectors, enabling the delivery of larger transgenes and simpler packaging of targeting constructs. LVs are integrating vectors that preferably integrate within active genes. They thus pose the risk of insertional mutagenesis that could lead to either the inactivation of endogenous genes, or oncogenesis stemming from insertional activation of proto-oncogenes. LVs can be modified to be integration-deficient (IDLVs) with a point mutation in the viral integrase protein, which removes the possibility of insertional oncogenesis. We have tested a strategy combining the benefits of LV delivery and the targeting accuracy of the CRISPR/Cas9 gene editing. To this end, we use a nicking Cas9 (Cas9n) and a single guide RNA (sgRNA) packaged to one IDLV, and a transgene template for homology-directed repair (HDR) and another sgRNA in a second IDLV. This dual IDLV delivery strategy minimizes the risk of off-target editing and ensures that the HDR repair template should be available at all double-strand breaks. In a proof-of-concept study, we delivered an EGFP transgene targeted to the *DAB1* gene in chromosome 1. Flow cytometry was used to quantitate transgene expression, while a junction PCR followed by Sanger sequencing was used to confirm the presence of homologous recombination events. Transduction of 293T cells with the dual transiently expressed IDLVs led to long-lasting EGFP expression resulting from homologous recombination of the transgene to the target site. An analysis carried out by droplet digital PCR showed that a fraction of the transduced cells contained small mutations in the genomic sequence between the sgRNA target sites, which is a known risk of genome editing. Our results show that the simultaneous delivery of IDLVs containing CRISPR/Cas9n and a transgene template can lead to targeted integration and sustained transgene expression. However, even at the lower mutation rate of Cas9n compared to the wild-type Cas9, the sgRNA selection requires care to minimise the risk of unintended editing events.

P0522

Identification of novel γ-globin repressors through a custom CRISPR knockout screen and validation of these repressors for the treatment of β-hemoglobinopathies

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Haemoglobinopathies, a group of conditions affecting haemoglobin, result from mutations in the HBB gene, leading to prevalent monogenic disorders such as β-thalassaemia and sickle cell disease. Reactivating the γ-globin gene for fetal haemoglobin (HbF) production emerges as a promising therapeutic strategy. However, current gene therapy approaches face limitations in terms of risks, costs, and accessibility. Additionally, pharmacologically targeting key regulators BCL11A and LRF (ZBTB7A) proves challenging due to their involvement in the regulation of non-erythroid genes. Identifying new factors amenable to pharmacological control is crucial for effective treatment of β-haemoglobinopathies.

Building on a prior custom CRISPR/Cas9 knockout screen targeting 293 genes, this study focuses on validating candidate genes associated with the screening phenotype, specifically HbF upregulation. Two candidate genes were selected for in-depth investigation. CRISPR/Cas-mediated knockouts were conducted through lentiviral transduction and nucleofection with ribonucleoproteins.

hCD34+ cells were isolated from peripheral blood using magnetic-activated cell sorting. Nucleofections involved two single and one double nucleofection per candidate gene, aiming to enhance disruption efficiency. Nucleofected cells were expanded, and erythroid differentiation spanned 11 days, with cell collection on the final day. HPLC analysis was used for assessing the levels of globins in the samples. Furhter, the HPLC analysis was quantified in order to detect the ratios of β/α and (Gγ+Aγ)/α.

The CRISPR/Cas-mediated knockdown of Gene A demonstrated a more pronounced upregulation of γ-globin compared to Gene B. Remarkably, employing a double nucleofection strategy for both Gene A and Gene B resulted in a more significant increase in γ-globin levels compared to the individual knockdowns. The observed increase in γ-globin levels with the double nucleofections highlights the potential for independent contributions of Gene A and Gene B to γ-globin regulation. Further dissection of their individual mechanisms is necessary for a comprehensive understanding of their roles in γ-globin modulation.

This project lays the groundwork for potentially validating new HbF regulators identified through a CRISPR-knockout screen. Ongoing investigations into the mechanisms of Gene A and Gene B, particularly their impact on HbF upregulation in hCD34+ cells, hold promise for identifying novel therapeutic targets. The outcomes may contribute to innovative strategies in treating β-haemoglobinopathies, offering significant advancements in genetic therapies for these inherited monogenic disorders.

P0523

Engineered ultracompact epigenetic editors for DNA and histone modifications enable durable epigenetic gene activation and suppression

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The rapid advancement in the discovery and characterization of epigenetic editors is paving the way for innovative therapeutic approaches to human genetic disorders. These developments are highlighted by the significant and enduring modulation of multiple therapeutically relevant genes,

both in vitro and in vivo, underscoring the promise of epigenetic editing. Despite these promising advances, two primary challenges impede the full realization of this potential: (1) the large size of current epigenetic editors for targeted DNA methylation and demethylation, which hampers effective tissue-specific delivery in vivo, and (2) the inconsistent modulation of gene expression by DNA methyltransferases and demethylases across different chromatin states and gene expression levels.

To address these challenges, we have engineered ultracompact yet highly effective DNA methyltransferase domains, along with compact and efficient DNA demethylase catalytic domains, and compact epigenetic gene activators. When combined with compact dCas proteins (<500aa), this new toolkit provides a versatile and potent platform for gene expression modulation. These novel efficacious engineered epigenetic suppressor tools are nearly 50% smaller than CRISPRoff, and novel DNA demethylase catalytic domains are 33% smaller than the conventional functional domains used for DNA demethylation. This results in a set of tools that can be packaged together with guide RNAs and tissue-specific promoters for delivery via AAVs for in vivo epigenome editing.

We demonstrate that these compact domains are functional in human cells and at endogenous human genes, thereby showing substantial potential for use in future epigenetic editing therapeutic payloads.

P0524

Characterization of a novel *TFAP2C* promoter

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TFAP2C is a key transcription factor controlling the differentiation of the trophectoderm layer and the subsequent formation of the placenta. Proper function of the placenta is important for the developing fetus by providing nutrient, waste and gas exchange and by protecting the fetus from maternal immune defense. Defects placental function can cause serious pregnancy complications such as miscarriage and preeclampsia. Embryonic *TFAP2C* expression is derived from a currently poorly annotated promoter that is active at the 8-cell embryo stage. The role of the new promoter in *TFAP2C* regulation is unknown, and the objective of this study is to shed light on its function and its role in the differentiation of trophoblast cells.

To investigate the regulation of *TFAP2C* we have created knock-out hESC lines for the novel *TFAP2C* promoter and the currently annotated *TFAP2C* promoter (consensus promoter). Additionally, we have set up inducible CRISPRa cell lines targeting the novel *TFAP2C* promoter and the consensus *TFAP2C* promoter. The outcome of the transcriptional perturbations of *TFAP2C* were quantified by immunocytochemistry and RT-qPCR.

Activation of the novel promoter lead to TFAP2C protein expression, even in cells with consensus promoter KO, confirming the function of the novel promoter. Interestingly, targeting of the novel promoter site for activation in KO cell for the same promoter still lead to TFAP2C protein expression, suggesting a role for the novel promoter in the regulation of the consensus promoter. Further experiments are ongoing for characterizing the role of the novel *TFAP2C* promoter in trophoblast differentiation.

The results of this work will provide insights into the dynamics of *TFAP2C* transcriptional regulation in early embryogenesis. Additionally, the work can aid in the robust production of

better trophoblast and placenta stem cell models. Developing proper placental cell models are necessary for understanding the mechanisms and developing new therapies for diseases related to the functioning of the placenta.

P0525

Towards double-strand break free gene silencing by miniature epigenetic editors suitable for AAV delivery

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CRISPR-associated (Cas) nucleases have become the dominant genome editing tool. First generation CRISPR-technologies involve introduction of double-strand breaks (DSB) at target specific DNA loci. However, lack of predictable editing outcomes and risks associated with DSB such as chromosome shattering (chromothripsis), translocations and large deletions have triggered the development of next generation CRISPR technologies which circumvent DSB. This includes epigenetic editing, which enables regulation of gene expression by epigenetic mark modifications instead of modifying the DNA sequence itself. State-of-the-art “CRISPRoff” induces persistent gene silencing by targeted gene methylation and histone modification. This makes it a promising candidate for hit-and-run gene silencing without continuous expression of the epigenetic editor (EE) in therapeutic settings. We established assays for epigenetic editing read-out utilizing CRISPRoff based on existing literature, and observed enduring silencing of the cell surface protein CD81 following a single hit-and-run treatment with the editor. Subsequent sequencing techniques, including next-generation sequencing and methylation sequencing, demonstrated that the promoter remained intact and exhibited high levels of CpG methylation. Numerous disease targets require in vivo delivery of EEs to distinct tissues for which adeno associated viruses (AAV) are considered a promising delivery vehicle. However, the large sized CRISPR nuclease *Streptococcus pyogenes* Cas9 (SpyCas9) and the additional epigenetic effector domains together exceed the AAV packaging limit, rendering it less well suited for therapeutic applications. The size requirements could be met by substituting SpyCas9 with hypercompact nucleases from the recently described type V-F family. To uncover their potential for EE applications, we screened publicly described Cas12f nucleases for CRISPR activation (CRISPRa) activity in HEK293T cells. However, compared to the described CRISPRoff construct based on SpyCas9, silencing activity was low or undetectable when SpyCas9 was replaced with alternative nucleases. Thus, further engineering and construct optimization seems required for EEs based on hypercompact nucleases.

P0526

Novel CRISPR/Cas9-based EDSpliCE system corrects splicing defect caused by the exonic variant *ABCA4* c.768G>T

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Bi-allelic pathogenic variants in ***ABCA4*** are causative of Stargardt disease (**STGD1**), an autosomal recessive retinal disorder, characterized by progressive loss of central vision and typically presenting during childhood. The onset of the disease is attributed to the accumulation of cytotoxic products in photoreceptors resulting from dysfunctional ABCA4 protein. Within the highly diverse mutational spectrum of *ABCA4*, the **exonic c.768G>T variant** represents a severe and frequent variant. This pathogenic mutation results in mis-splicing by inducing a 35-nucleotide elongation of the exon 6 that is retained in the mature mRNA transcript. This is due to the weakening of the canonical donor splice site and the presence of a strong cryptic splice donor site located 36 nucleotides downstream. Consequently, a frameshift occurs, leading to the formation of a truncated protein, p.(Leu257Valfs∗17).

CRISPR/Cas-mediated genome editing strategies have been shown to effectively correct splicing defects restoring proper mRNA processing and protein translation. This provides a potential and permanent solution for genetic defects caused by splicing-affecting variants. Nevertheless, no CRISPR/Cas9-mediated strategies have been established to correct faulty splicing due to pathogenic variants located near exon/intron boundaries. To address this challenge, we used our novel gene editing platform Enhanced-Deletion Splicing Correction Editing (**EDSpliCE**) to successfully correct the splicing defect caused by the c.768G>T variant.

A mutant (MT) minigene (MG) plasmid containing the *ABCA4*:c.768G>T variant was cloned. The induction of pathogenic splicing was confirmed through minigene assay in HEK293T. The validated MT-MG was then co-transfected with different individual single guide RNAs (sgRNA) and the Cas9-ortholog EDSpliCE. The percentage of correctly spliced transcripts was calculated through mRNA splicing analysis, followed by PCR reaction analysis using chip electrophoresis as a readout. Editing efficiency and profile were analyzed by targeted high-throughput sequencing (HTS). To study the potential of the top-sgRNAs in the correction of the splicing defect in photoreceptor precursor cells (PPCs), an isogenic homozygous *ABCA4*:c.768G>T-induced pluripotent stem cell (iPSC) line was generated by direct delivery of CRISPR/Cas9 system as a ribonucleoprotein (RNP) complex along with a donor template. Once characterized, iPSCs were differentiated into PPCs and will be used for AAV-mediated delivery of the established EDSpliCE editing strategies.

Our results demonstrate that the EDSpliCE system can effectively address pathogenic splicing variants at exon/intron boundaries by inducing directional and enhanced perturbations of the sequences involved in mis-splicing. EDSpliCE achieved up to 85% correction rate of the c.768G>T-induced splicing defect at the minigene level, outperforming the limited rescue seen with the parental wild-type Cas9 system. This positions EDSpliCE as a promising approach with significant potential for addressing aberrant splicing variants, including those located in exonic regions near exon-intron boundaries. Furthermore, our findings demonstrate a significant therapeutic potential of EDSpliCE for correcting the mis-splicing caused by the *ABCA4* c.768G>T variant associated with STGD1.

P0527

Development of Prime Editing targeting the R14del Phospholamban in cardiomyocytes

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In the Netherlands, 12% of patients diagnosed with arrhythmogenic cardiomyopathy (ACM) and 15% of patients with Dilated Cardiomyopathy (DCM) have proven to have a mutation in the PLN gene leading to the loss of an Arginine in position 14. These numbers make of the R14del Phospholamban the most prevalent cardiomyopathy-related mutation. Gene therapy and gene editing are tools constantly being developed. The latest tool in gene editing is Prime Editing developed by Pr. David Liu from Harvard in 2019. This tool fusions a mutated Streptococus Pyogenes Cas9 with a nickase activity to a Reverse transcriptase. This construct complexed with a guiding RNA, can target a genomic locus, and proceed with virtually any genomic mutation. The Nickase activity allows for single strand nick avoiding the double stranded DNA induced by the normal CRISPR Cas9 system. A modified 3' ending of the Prime editing guiding RNA (PegRNA) can then anneal to the genomic DNA at the specific targeted locus in the genome. This segment is called the Primer Binding Site (PBS). After the annealing, an overlapping RNA segment called the Reverese Trancriptase Template (RTT) will then be Reverse transcribed into DNA which will contain the desired edit. The main goal of this project is to correct the PLN R14del mutation through the use of this novel technique. Edit efficiency is locus dependent but not only as the length ratio of the PBS and RTT can have an impactful effect on the overall edit efficiency. In this first part of the study, the focus was set on investigating the effect of the nucleotide ratio (PBS/RTT) on the edit efficiency for the PLN on the HEK293 cells. The efficiency is studied through ddPCR after Lipofection. 5 different sizes of PBS have been investigated from 10 to 15 nucleotides and 5 diferent sizes of RTT spanning from 15 to 24 nucleotides for the RTT with a 13 nucleotide PBS. The initial results show an effect up to 10% of edit and no real effect on different sizes of RTT. Moreover, with this initial study we confirm the effect of different sizes of PBS. 14 and 15 nucleotides are the best nucelotides sizes for the PBS. Further study will include the analysis of GFP positive cells to see the actual edit efficiency on positively selected cells through FACS. The DNA extracted from the cells can then also be sent for sequencing. Once the most optimal construct has been selected, the study will be pursued on cardiomyocytes presenting the PLN R14del mutation.

P0528

Single-cell assessment of genome integrity and toxicity events associated with edited cell therapies

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Advances in cell and gene therapy are revolutionizing the treatment and potential cure of diseases such as cancer and rare genetic disorders. Precision gene editing technologies like CRISPR-Cas9, TALENs, and ZFNs have surpassed traditional methods, enabling new strategies to correct genetic defects at their source. However, despite these advancements, the editing process yields heterogeneous populations where some cells may have undesired outcomes that bear the risk of genome toxicity leading to potential malignancies. These adverse effects can include the introduction of off-target edits, copy number variations, and chromosomal aberrations like translocations or large indels. Consequently, the success of effective gene therapies depends on the ability to accurately measure and understand these events. Furthermore, since "cells" are the functional units of gene editing products, it is prudent to measure the co-occurrences of editing results and potential genotoxicity events in a single-cell context.

In this study, we demonstrate the use of an advanced single-cell technology that combines microfluidics and multiplex PCR to simultaneously measure several critical aspects of gene editing.

The assay analyzes the co-occurrence and zygosity of on-target edits, off-target edits, quantitatively detect translocations between predicted edit sites, and map the genomic copy number variation (CNV) landscape, all within over 10,000 cells in parallel. Due to the assay's single-cell resolution, this method delivers a detailed and comprehensive view of the heterogeneous editing profiles present in gene-edited products. It allows for precise and rapid evaluation of both the editing outcomes and the overall genomic integrity, facilitating the identification of potential malignant events and improving the safety and efficacy of the gene therapy.

To evaluate the robustness of this workflow, we utilized a variety of samples with different levels of adverse effects from multiple gene editing experiments. These samples were validated orthogonally using bulk NGS (DNA-seq), rhAMPseq, and whole-genome sequencing for CNV. Employing a targeted sequencing panel (611-plex), in a single assay, we measured on- and off-target editing activity, detected structural variations, identified gene editing-induced translocation events, and assessed genome-wide CNVs at a single-cell level in these samples. The resulting data were analyzed using a novel bioinformatics pipeline specifically developed to simultaneously and accurately measure all these modalities. Additionally, for locus-level CNV detection, we demonstrated the performance of the single-cell CNV assay at a focal level. This was exemplified by confirming a Chr20q copy number alteration in an iPSC cell line, which conventional methods such as G-banding and CMA failed to detect.

This high-throughput single-cell NGS assay represents a significant advancement in gene editing analysis, measuring both intended and unintended consequences of genome editing across a variety of resolutions. This dual functionality not only aids in optimizing gene editing protocols but also represents a streamlined and thorough process for assessing the safety aspects of gene-modified therapies.

P0529

Base editing as a therapeutic strategy for autosomal dominant tubulointerstitial kidney disease

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Autosomal Dominant Tubulointerstitial Kidney Disease (ADTKD), most commonly caused by monoallelic gain-of-function missense variants in UMOD (encoding Uromodulin), is the third most common cause of monogenic kidney disease in adults. Pathogenic variants in UMOD induce a toxic gain-of-function effect, probably due to protein misfolding and subsequent endoplasmic reticulum (ER) retention, resulting in ER stress and unfolded protein response. This eventually gives rise to interstitial fibrosis and tubular atrophy. To date, dialysis and kidney transplantation are the only therapeutic options upon kidney failure. Therefore, novel therapeutic options for ADTKD patients are urgently needed. DNA base editors, offering high efficacy and easy programmability, represent a promising new treatment strategy. This project aims to establish renal base editing for the preventive treatment of ADTKD.

Systematic screening of suitable guide RNAs (gRNAs) and base editors for correcting the pathogenic human UMOD variant C170Y in engineered HEK293T cells resulted in on-target editing efficiencies of up to ~75%. In total, 12 combinations of gRNAs and base editors were

tested, resulting in variable degrees of on-target (~6.5%-79.3%) and bystander editing (0.0%-68.0%) as determined by deep sequencing of the targeted region. Potential gRNA-dependent off-target editing was assessed *in silico* using Cas-OFFinder. Subsequent base editing in a mouse inner medullary collecting duct (mIMCD-3) cell line expressing the human C170Y variant attenuated the disease phenotype by reducing ER retention of UMOD and restoring physiological trafficking to the cellular membrane. Western blotting further validated successful phenotypic rescue, indicated by an increase in the fraction of mature, fully Golgi glycosylated Endo-H insensitive Uromodulin in mIMCD-3 cells after base editing, from 0% in the unedited control to ~20% in the edited samples – comparable to the ~30% mature UMOD found in the corresponding wild-type cell line. Furthermore, the disease phenotype of the C170Y mutation could be reproduced in human urine-derived renal epithelial cells (hURECs), where the UMOD-C170Y localised to the ER.

An additional approach, independent of the causative pathogenic variant, is based on the observation that Umod-/- mice and humans carrying monoallelic truncating variants lack an obvious renal phenotype. We tested UMOD inactivation by introducing premature STOP codons. In HEK293T cells, we achieved up to ~80% on-target editing for this approach. Functional evaluation of knockdown through western blot revealed that UMOD was decreased after editing.

We will validate our different approaches using human renal organoids derived from patients affected by ADTKD-UMOD. These patient-derived organoids may serve as a novel platform for modelling ADTKD-UMOD *in vitro*, for both exploring novel treatment approaches and characterising disease mechanisms. In summary, we anticipate base editing to serve as an effective therapeutic approach for monogenic kidney diseases.

P0530

Optimization of the modular Pin-point™ base editing platform with an AI-engineered CRISPR-Cas effector

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The application of language models and artificial intelligence (AI) in protein design represents a transformative approach in the discovery of novel proteins. This approach offers significant promise, particularly in the gene editing field where naturally occurring enzymes that work well in mammalian systems are limited. Traditional methods of protein engineering often rely on labour-intensive and time-consuming processes such as random mutagenesis, DNA shuffling and directed evolution. In contrast, AI-driven models, inspired by natural language processing techniques, can enable the generation of novel proteins with optimal functional properties bypassing evolutionary constraints. Profluent's OpenCRISPR-1 is the first AI-generated gene editor that has been released to the market, demonstrating editing capacities similar to those of SpCas9.

Base editors were first described in 2016 as a promising next generation genome editing tool that has rapidly progressed to clinical applications. Revvity's Pin-point base editing platform is a modular system which in some configurations consists of either a nuclease-deficient or nickase Cas enzyme, an aptameric guide RNA (sgRNA), and a deaminase fused to an aptamer binding protein. These components assemble within the cell and act in concert to facilitate precise single-nucleotide conversions. The inherent modularity of the Pin-point platform allows for the swapping of nuclease and deaminase components to achieve optimal editing results. Here, we demonstrate robust editing by incorporating a nickase OpenCRISPR-1 enzyme into the Pin-point

base editing platform. The Pin-point editing machinery in this example is tailored for base editing applications to therapeutically relevant cell types such as T cells and induced pluripotent stem cells (iPSCs). Using base editing to induce gene knockout, we targeted four therapeutically relevant genes for the development of allogeneic CAR-T cells, achieving high editing efficiency at all targeted sites simultaneously. The modular nature of the Pin-point system offers the unique advantage of enabling simultaneous site-specific knock-in of a CAR using aptamer-less gRNA while performing base editing on other sites using aptamer-containing gRNAs limiting the drawbacks of sequential delivery, gRNA crosstalk, or the requirement of orthogonal Cas enzymes.

By having successfully integrated the open-access nuclease OpenCRISPR-1 with our innovative Pin-point base editing platform, we demonstrate the versatility of our platform and provide further choices when applied to advanced gene editing technologies. This combination shows high levels of base editing precision and efficiency in a therapeutic context and exemplifies a significant leap forward in the development of tools for cutting-edge genomic medicine.

P0531

Recombinant nuclease Cas9 for therapeutic genome-editing – the GMP manufacturer's point of view

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Genome editing with the CRISPR/Cas method has revolutionized molecular-biologic methods and the resulting therapeutic approaches. Nuclease Cas9 is an essential component of a recently market-approved therapy against sickle cell disease (SCD) and transfusion dependent beta-thalassemia (TDT). In this case study, we present an overview on the corresponding manufacturing campaign to supply those therapeutic programs with recombinant nuclease spCas9 under the requirements and conditions of current Good Manufacturing Practice (cGMP).

Cas9 was expressed as a recombinant protein by using IPTG-induced *E. coli* culture as production system (pET/T7 promoter). A manufacturing process was developed and optimized, essentially consisting of fed-batch cultivation, high-pressure cell disruption, a three-step chromatography regime, formulation by ultra/diafiltration and final aseptic filling.

Therapeutic nucleases for genome editing have to fulfil high and defined quality standards with respect of functionality/activity, homogeneity and purity. A set of analytical assays for routine in-process control and final quality control was developed and validated, and a respective list of specifications was defined. In addition, extended biochemical characterization of Cas9 was performed. Particular emphasis was put on the determination of the activity of Cas9 by application of an in-vitro potency assay. With respect to homogeneity, Cas9 aggregates and truncated variants thereof were characterized by high-performance size exclusion chromatography (HP-SEC).

Long-term storage stability was shown to be sufficiently given by conducting multi-year stability studies. By performing forced degradation studies, the impact of extreme physico-chemical conditions on Cas9 potency was evaluated.

For market submission, the performance of a manufacturing process is required to be validated. Exemplary data will be shown on critical quality attributes (CQA), down-scaled process

characterization studies and their evaluation by statistical design of experiments (DOE). Finally, process validation was performed by conducting consecutive, full-scale and cGMP conform runs for process performance qualification (PPQ).

In this comprehensive overview, the concepts, difficulties and results of late-stage clinical / pre-marketing manufacturing of GMP-grade recombinant Cas9 will be discussed.

P0532

Systematic Genomic Safety Assessment of CRISPR-Based Gene Therapy in Muscle Stem Cells

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Limb girdle muscular dystrophies (LGMD) are monogenic diseases that severely reduce the life quality of patients, with potentially fatal outcomes. We have established CRISPR-based gene correction in primary human muscle stem cells (MuSCs) *ex vivo* for common LGMD-causing mutations. A major concern of the CRISPR-based gene correction is the potential genome-wide introduction of harmful mutations. Here, we elaborated a systematic evaluation of genome integrity in gene-edited MuSCs. As part of this evaluation, we established a sensitive and reproducible GUIDE-seq protocol in MuSCs. We identified the off-targets of candidate sgRNAs targeting three LGMD-causing mutations in *SGCA* and *CAPN3*, and one MD-causing mutation in *DMD*. These experiments uncovered a frequent off-target mutation in the tumor suppressor gene *CUL9*, which could be suppressed by a shift in the respective sgRNA. Moreover, a non-predicted off-target was identified with high GUIDE-seq read numbers and confirmed by deep sequencing. For most predicted and low-read GUIDE-seq off-target sites, multiplex deep sequencing showed low or no off-target editing in the MuSCs edited with the gene editing protocol we established for the clinical application. Tumor panels and preliminary translocation analysis did not reveal any large-scale genomic aberrations in the Cas9-edited MuSCs. Overall, these results underline the importance of a thorough genetic safety assessment prior to clinical trials.

P0533

Efficient and specific genome editing with Metagenomics-derived base editor for human therapeutic applications

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Base editors (BEs) are powerful gene editing tools that are leveraged to knock out genes through single base transitions, enabling multiplexed gene editing without risk of translocations. However, broad implementation of BEs for cell therapy is challenging due to the limited targetability of SpCas9 adenine base editors (ABEs) and the significant cell toxicity observed with cytosine base editors (CBEs). Previously, we have described an efficient metagenomics-derived ABE composed of a PAM interacting domain-swapped chimeric nickase and evolved deaminase, which we validated in vivo. Here we present highly efficient and specific multiplexed base editing with the chimeric ABE, including an all-in-one engineering approach using 29-1, a Cas12a nuclease, to simultaneously and efficiently knockout TRAC and knock in a chimeric antigen receptor (CAR) for allogeneic cell therapy applications. Using the ABE, we achieved effective and reproducible multiplexed protein knockdown in primary T cells of CIITA-29, β2M-1, and PDCD1-2 over 98%, with minimal InDels. Triplex protein knockdowns were durable without impacting cell viability or expansion. No detectable translocations and no significant genomic composition differences were found when compared to unedited cells. The gene expression profile of ABE multiplexed cells show no increased IFN α and γ response or p53 activation, in contrast to SpCas9 nuclease and BE edited cells, which have shown decreased engraftment efficiency in vivo correlated to stress response gene activation. Finally, to determine if we could simultaneously knock out TRAC and knock in a CAR construct in addition to the ABE multiplex, we electroporated ABE with 29-1 and CD19-CAR nanoplasmid for the TRAC locus knockin. We observed equally efficient multiplexed ABE KO, 95% TRAC protein knockout, and >40% CD19-CAR integration, with stable long-term CAR expression and CAR-T potency and improved cell viability compared to viral cargo integration. These results demonstrate how highly efficient and specific chimeric base editors with greater cell tolerability and targetability, in combination with a novel nuclease with highly efficient gene integration properties, enable a potent all-in-one editing solution for the development of next generation cell therapies. Given the successful application to cell engineering applications, these editing systems will also be enabling tools for developing in vivo genetic medicines.

P0534

Exploring therapeutic efficacy of base editing for clinically relevant pathogenic variants associated with Autosomal Dominant Polycystic Kidney Disease

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Autosomal Dominant Polycystic Kidney Disease (ADPKD) is the most common hereditary nephropathy, responsible for 8-10% of cases of end-stage renal disease. It is hypothesised that a single inherited mutation in the PKD1 or PKD2 gene is insufficient to trigger disease manifestation. Instead, the development of cysts and the onset of the disease are believed to require a "second hit" somatic mutation in either PKD gene. Their induced re-expression can result in at least partial recovery of kidney function and a reduction in phenotypic features of ADPKD. This suggests a potential for ADPKD treatment through gene editing methodologies. Particularly interesting in this background are base editors, catalytically dead Cas9 proteins fused to a deaminase and guided by a single guide RNA (sgRNA), can precisely convert specific bases in the genome without introducing double-strand breaks. Notably, the first base editing therapies are currently being tested in human trials.

This project explores the potential of base editing to correct pathogenic variants causing ADPKD *in vitro*. Of the 187 pathogenic variants from patients presenting to the Charité Center for Rare Kidney Diseases, 104 were identified as point mutations, and 67 were selected for experimental assessment. Criteria for inclusion were based on suitable PAM-sites (NGG/NG), single nucleotide polymorphisms (C>T, G>A, T>C, and A>G) and the position of the target base within the editing window of the base editors (BE4max & ABE8e). 150 bp of genomic sequence surrounding the pathogenic variants were integrated into HEK293T cells using a cut-and-paste mechanism mediated by a transposase enzyme. Combinations of base editors and guide RNAs were transfected into the cells followed by targeted amplicon sequencing. We observed varying degrees of editing precision and efficiency, reaching up to 71%.

Moreover, it was investigated whether mutation correction could reverse the associated protein and functional impacts. This was tested using a specific mouse mutation, located in the REJ domain, which leads to partial defect in PC1 cleavage, a crucial process for maintaining its cellular physiological functions. The mutation therefore leads to increased ER stress, as measured by sXBP1 expression. Using western blot, it could be observed that mutant cells show no Polycystic 1 (PC1) with increased levels of sXBP1 expression compared to wild type. This effect was attenuated upon base editing. In HEK293T cells, the introduction and subsequent correction of ADPKD mutations were successfully achieved in a significant proportion of the tested mutations, showcasing the versatility and potential of these base editors. For all targets, off-target analysis will be conducted using Cas-OFFinder.

Our findings establish a method for testing base editors as a therapeutic strategy for ADPKD. We demonstrate mutation correction efficiencies of up to 71% and show that correction can lead to a significant reduction in ER stress. Overall, this study provides a valuable framework for optimizing and testing the functionality of patient-specific gene therapy *in vitro*.

P0535

An optimized and validated workflow for developing stable producer cell lines with >99.99% assurance of clonality and high clone recovery

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There is constant pressure to reduce timelines in mammalian cell line development (CLD) for biotherapeutic protein production. Demonstration of clonal derivation of the generated cell lines is key for health authorities' approval. To meet these regulatory and process-oriented demands, single-cell dispensers and plate imagers are vital to any CLD laboratory. Here we present the UP.SIGHT 2nd Gen that combines single cell dispensing and plate imaging capabilities.

Single cell dispensing experiments were performed to determine single-cell dispensing efficiency (SCDE) and the probability of clonal derivation (p(clonal)). Cell growth and monoclonal antibody titer quantification were determined using the UP.SIGHT 2nd Gen's imaging capabilities. The data was analysed using the C.STUDIO 2.0 software.

The instrument's SCDE resulted in 97.81% and (p(clonal)) was higher than 99.99%. Furthermore, we show that the cloning efficiency can be up to 80% when working with optimized medium conditions. Importantly, we also demonstrate that growth assessment via well bottom imaging or

cell counting and titer measurement of monoclonal antibody-like molecules can be performed on the UP.SIGHT 2nd Gen.

Data analysis in C.STUDIO 2.0 guides the user to select the best clones and provides comprehensive data logs and reports for IND/BLA submissions.

UP.SIGHT 2nd Gen enables fast and efficient CLD workflows, covering all the steps from single-cell dispensing with assurance of clonality to colony tracking and titer measurement. This ultimately results in better documentation for the improved quality of the final biological product.

P0536

Establishment of Adult Cardiomyocyte-specific Oga Knockout Mouse Model using CRISPR / Cas9 System

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Constructing an adult cardiomyocyte-specific Oga knockout mouse model to study the function of endogenous OGA in the maintenance of adult cardiac homeostasis. Firstly, Screening the effective sgRNAs targeting the Oga coding region and and constructed them in AAV9 virus. Then AAV9 was injected into cardiomyocyte specific SpCas9 knockin mice (α-MHCCas9) by intraperitoneal injection. qPCR and Western blot were performed to confirm whether Oga was successfully knockout. Finally, to study the function of Oga in cardiac homeostasis maintenance, the cardiac structure was analyzed by histology and the cardiac function was analyzed by transthoracic echocardiography. Two effective sgRNAs targeting Oga were identified and delivered by AAV9 to disrupt Oga gene successfully in cardiomyocytes. Consistently, O-GlcNAcylation level was significantly increased by Oga deletion. There were no significant differences in overall structure and function indicated by the histological and echocardiographic analyses. By using the AAV9-mediated delivery of CRISPR / Cas9 system, we successfully constructed the adult cardiomyocyte- specific Oga knockout mouse model, which would be provided as an effective mouse model for studying the function and mechanism of OGA-mediated O-GlcNAcylation clearance in the maintenance of cardiac homeostasis.

P0537

Development of a new gene editing strategy for gain-of-function SAMD9L mutations

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Germline predisposition has been recognized as an underlying cause of bone marrow failure (BMF) and myelodysplastic syndromes (MDS) in children. These disorders, characterized by a

heterogeneous group of clonal hematopoietic stem and progenitor cell (HSPC) abnormalities, lead to impaired hematopoiesis and may progress to leukemia. SAMD9L, a newly identified gene associated with BMF/MDS risk, has been linked to inflammation and the anti-viral stress response. Most patients carry gain-of-function missense mutations in SAMD9L, which inhibit protein translation, leading to rapid cell death and impaired hematopoiesis.

Given the limitations and risks associated with hematopoietic stem cell transplantation, there is an urgent need for innovative treatment approaches. Conventional lentiviral-based gene therapy is not feasible for most BMF/MDS genes due to toxicity of uncontrolled overexpression, TP53 activation and insertional mutagenesis risk. Base editing (BE) using adenine base editors (ABE) and cytosine base editors (CBE), which enable precise A-to-G or C-to-T nucleotide transitions, along with prime editing (PE), are advanced gene editing techniques without DNA double-stranded breaks, offering a promising therapeutic alternative.

We developed a gene editing strategy targeting SAMD9L mutations. Initially, we established a comprehensive database encompassing the world's largest cohort of SAMD9L patients (n=187). Our analysis revealed that 47.1% (88/187) of mutations are amenable to correction using ABE, 20.3% (38/187) using CBE, and the remaining 32.6% (61/187) using PE. We designed an innovative lentiviral (LV) minigene model in the HEK293T cell line to assess base editing efficiency for the six SAMD9L mutations: c.2956C>T, p.(Arg986Cys); c.2957G>A, p.(Arg986His); c.3353A>G, p.(Tyr1118Cys); c.4534G>A, p.(Val1512Met); c.3842G>A, p.(Arg1281Lys) and c.1877, p.(Ser626Leu). Focusing initially on the most common c.2956C>T mutation, we identified an efficient single guide RNA (sgRNA), which, in combination with the ABE8e-SpRY protein (a SpCas9-derived variant with near-PAMless recognition capabilities), achieved 35% on-target efficiency, as measured by NGS 6 days post-electroporation. Transitioning to patient-derived cells, we successfully generated induced pluripotent stem cells (iPSCs) and T cells from the peripheral blood of the same patient. On-target correction efficiencies were 2.0% in T cells and 7.2% in iPSCs for minigene pre-screened sgRNA. The high frequency of bystander nonsynonymous edits (up to 21%) prompted the exploration of prime editing for enhanced precision, which is currently in progress. Screening additional mutations in patient-derived models using sgRNAs in combination with ABE8e-SpRY or ABE8e-SpCas9-NG yielded on-target editing efficiencies of 16.7% (c.2957G>A), 35.6% (c.1877C>T), and 20.0% (c.3842G>A). Concurrently, we are conducting hematopoietic differentiation into myeloid and erythroid linages to assess phenotypic evidence of mutational compensation (on-target vs bystander) and to determine how these mutations influence cell fitness.

In summary, our minigene LV system facilitates efficient pre-screening of sgRNAs for SAMD9L mutations, with these pre-identified sgRNAs also demonstrating editing capability in patient-derived iPSCs and T cells, albeit at lower efficiency. This approach, utilizing both artificial and patient-derived cellular models, advances personalized medicine strategies in SAMD9L disorder and other hematological diseases.

P0538

Detection of G6PC RNA and protein activity in serum exosomes as pharmacodynamic biomarkers for GSDIa *in vivo* gene editing therapy

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Glycogen Storage Disease Type Ia (GSD1a), also known as von Gierke disease, is a metabolic disorder characterized by severe hypoglycemia associated with the accumulation of glycogen and fat in the liver and kidney. It is caused by mutations in the G6PC gene encoding the catalytic subunit of glucose-6 phosphatase (G6Pase), a predominantly liver-expressed enzyme catalyzing the hydrolysis of glucose-6-phosphate (G6P) to glucose and phosphate. The pathogenic variant G6PC-p.Arg83Cys (R83C) is a prevalent variant associated with severe manifestations of GSDIa, which results in life-threatening fasting hypoglycemia as well as long-term complications impacting the liver and kidney, without appropriate management. Lipid-nanoparticle (LNP) mediated *in vivo* gene editing allows direct editing within the body. BEAM-301 is a liver-targeting LNP formulation containing gRNA and mRNA components optimized to correct the R83C mutation. The G6PC protein is not secreted from the liver into the circulation, and therefore, poses a challenge to assess pharmacodynamic activity post-treatment. Exosomes, are nano-sized extracellular vesicles actively secreted by various cell types and are abundantly present in many body fluids, including serum. Exosomes contain proteins, microRNAs (miRNAs), and messenger RNAs (mRNAs) that reflect the conditions of their donor cells and, therefore, are ideal biomarkers. Here we report a method that utilize exosomes from serum for detecting the base editing in RNA and confirming the restoration of G6PC protein activity post-treatment in the liver.

To obtain exosomes with wild-type G6PC, blood samples were obtained from healthy donors (n>10) with normal G6PC activity using serum separation tubes, followed by serum preparation using standard protocol. Exosomes were then isolated from the serum. For comparison, liver microsomes were isolated from G6pc-R83C transgenic mice with no G6PC activity. RNA and total protein were isolated using protocols optimized at Beam. To detect the mutation in exosomal RNA, cDNA was reverse transcribed from RNA followed by ddPCR SNP assay using fluorescent tagged probes specifically targeting the R83C mutation or wild-type nucleotide. To measure the G6PC enzyme activity, a phosphohydrolase assay was performed and the activity was normalized to total protein measured by BCA (Bicinchoninic acid) assay.

In summary, significant improvement in RNA yield was achieved through our optimized RNA isolation protocol. We specifically detected the wildtype single nucleotide in the exosomal RNA obtained from the healthy human donor. Additionally, the G6PC activity was detected and quantified in serum exosomes isolated from healthy donors, while no activity was observed in liver microsomes isolated from G6pc-R83C transgenic mice.

Collectively, our approach highlights the potential utility of exosomal RNA and proteins as non-invasive pharmacodynamic biomarkers to assess response to treatment in *in vivo* base editing therapy for GSD1a.

P0539

Drug metabolism and pharmacokinetics in mice systemically administrated with a base editing drug for Duchenne Muscular Dystrophin (DMD)

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DMD is a progressive muscle-wasting disease caused by mutations in the gene encoding the dystrophin protein. Until now, there is no effective drug for DMD. GEN6050X is an intravenously cytosine base editing drug for DMD exon 50 amenable patients. GEN6050X contains two AAV9 vectors, ss.AAV9.oTAM and ss.AAV9.hE50-sgRNA. In DMD patients amenable to exon 50 skipping, GEN6050X can restore the dystrophin protein through inducing exon 50 skipping. GEN6050X also carries a weak therapeutic gene ACTG1, which can provide a synergetic effect with *de novo* synthesized Dystrophin protein. The understanding of GEN6050X Drug metabolism and pharmacokinetics (DMPK) will facilitate the interpretation of toxicity and prediction of in vivo efficacy of base editors.

In this study, we investigated the dynamics of vector DNA and transgene expression in wild-type B6 mice after a tail vein injection with 2E14 vg/kg GEN6050X for 3 months.

After administration, the two vectors level of GEN6050X in blood fell rapidly in mice. On Day 8, oTAM and sgRNA vector DNA decreased by 99.54% and 99.52% of the level of Day 1. The half-life of both components is 1.7 day.

The oTAM and hE50-sgRNA vectors were widely distributed in the tested tissues on Day 1. Since Day 8, the homeostatic tissue biodistribution of vector DNA was formed with predominant distribution in liver, adrenal gland, heart and injection site. The vector DNA exhibited different elimination rates in these four tissues. In liver, oTAM and hE50-sgRNA vectors DNA fell rapidly from Day 8 to Day 22 followed by slow elimination to Day 92. The half-life of oTAM and hE50-sgRNA in liver were 44.1 and 35.5 days. In heart and adrenal gland, vectors DNA maintained stable between Day 8 and Day 22, then slowly descend to Day 92. The oTAM and hE50-sgRNA half-life is 71.0days and 73.3 days in heart while 36.5 and 30.7 days in adrenal gland, respectively. From Day 8 to Day92, the vectors DNA in injection site and other low-distributed organs declined slowly with oTAM/ hE50-sgRNA half-life ranging from 26 to 73.3 days. No gender difference of elimination pattern in all tested tissues was observed. On Day 92 after administration, the vectors DNA is predominantly distributed in liver, followed by heart, adrenal gland, injection site and other tissues.

In mice dosed with 2E14 vg/kg GEN6050X, low-level of hE50-sgRNA transcription began to appear in the heart, liver and injection site of mice on day 8. Since day 22, hE50-sgRNA transcripts were predominantly distributed in the heart. Cardiac hE50-sgRNA transcript levels peaked on day 22 (127700 copies/ ng total RNA) and remained stable thereafter until day 92 (106650 copies/ ng total RNA). After the liver and injection site peaked on day 8, transcript levels continued to decline with half-life of 74.7 and 112.3 days in liver and injection site. The analysis of oTAM and ACTG1 protein in tested tissue is ongoing.

In conclusion, the up-to-date DMPK data of GEN6050X demonstrated the vector DNA will eliminate over time after administration and the transcription of sgRNA showed different dynamics with vector DNA.

P0540

A novel, compact CRISPR/Cas13 with high efficiency and fidelity for genome editing therapy

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CRISPR/Cas13 systems have emerged as powerful tools for precise manipulation of RNA, offering a compelling therapeutic avenue for introducing temporary, non-heritable modifications – ideal when transient changes are preferred or DNA editing poses technical challenges. Despite their promise, translating Cas13-mediated RNA editing into clinical applications faces hurdles, chiefly due to the large size of existing Cas13 editors, which exceed the cargo limit of AAVs, the prevalent vectors for gene therapy. Moreover, the inefficiencies and lack of precision in accurately targeting specific transcripts with current Cas13 tools further complicate their therapeutic use. To address these impediments, we embarked on a search for novel Cas13 effectors by exploring huge microbial genomes. This endeavor led to the discovery of a groundbreaking compact Cas13 enzyme, named CasRm.

CasRm, with only 893 amino acids, overcomes the size limitation for AAV delivery. Its compactness aside, CasRm exhibits extraordinary editing proficiency in comparison to conventional Cas13 enzymes, when tasked with the manipulation of various endogenous genes including *Vegfa*, *Ptbp1*, *Pcsk9*, *Aqp1*, *Ctgf*, *Ar*, *Mitf*, and *Srd5a2*. Notably, CasRm outshines RfxCas13d, a prevalent Cas13 variant, and significantly eclipses the performance of PspCas13b, Cas13X.1, and Cas13Y.1. Typically, Cas13 activities can lead to off-target effects or unspecific RNA cleavages, causing the downregulation of numerous genes. To assess this, we conducted RNA sequencing to identify differentially expressed genes upon targeting *Egfp*. In contrast to non-targeting controls, CasRm resulted in the downregulation of a minimal number of genes, whereas other Cas13 variants triggered downregulation of a significantly larger gene set. This observation strongly implies that CasRm possesses a strikingly low level of off-target activity.

To validate the therapeutic potential of CasRm, we advanced its application in an AAV-mediated RNA targeting approach focused on *Ptbp1* a key player in Parkinson's Disease (PD) pathology. Our findings revealed that CasRm successfully downregulated PTBP1 expression and induced astrocyteto-neuron transdifferentiation that could significantly improve the motor function of PD animals. Furthermore, we assessed the efficacy and safety of a CasRm-based therapy in treating CNV, a condition associated with nAMD, by knocking down *Vegf* in non-human primates. CasRm effectively reduced VEGF levels, inhibited laser-induced CNV formation, and was shown to be well-tolerated. Collectively, these results affirm CasRm's competence in fulfilling the requirements for in vivo editing and its potential deployment as a therapeutic strategy.

In conclusion, CasRm stands as a groundbreaking, compact iteration of the Cas13 family, distinguished by its exceptional combination of high efficacy and precision for both in vitro and in vivo RNA editing applications. Its remarkable editing capabilities establish CasRm as a highly adaptable instrument with broad applicability across the landscape of RNA modification and gene therapy.

P0541

Identification of potential megakaryocytic lineage-specific regulatory regions using the CRISPR/dCas9-KRAB system for targeted gene therapy applications in inherited platelet disorders

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Glanzmann Thrombathenia (GT) is an inherited platelet disorder (IPD) associated with severe bleeding due to congenital mutations that impair platelet functionality. These alterations affect the patients from birth, with a prevalence of 1 in a million in the general population, but as high as 1 in 200,000 in certain areas or ethnic communities. In GT, the mutations occur in the *ITGB3* and *ITGA2B* genes, which form the αIIbβ3 integrin responsible for the proper development of stable thrombi and blood clot retraction. Currently, the only treatments for IPD involve the use of hemostatic drugs to prevent bleeding or allogenic hematopoietic stem cell transplantation. Therefore, there is a need for novel targeted therapies for IPD.

In recent decades, the use of autologous hematopoietic stem and progenitor cells (HSPCs) coupled with gene therapy protocols has been established as a feasible alternative. However, several challenges must be overcome before gene therapy becomes widely available for patients with IPDs. One of the major drawbacks is the need for a regulated transgene system; constitutive overexpression could lead to unwanted side effects (biased differentiation, cell cycle interference, …). Therefore, we aim to characterize MK-specific promoters to drive transgene expression.

To address this need, we employed CRISPR interference (CRISPRi) tiling screening for the functional profiling of candidate regulatory elements. This system enables the identification of potential lineage-specific promoters and gene-specific enhancers that can be used in novel therapies for restoring gene-corrected expression. Initially, MK-specific genes were screened using a series of in silico-based approaches to identify such specific promoters. Additionally, we developed two megakaryocyte-derived cell lines (MEG-01 CRISPRi-Puro and MEG-01 CRISPRi-mCherry) that constitutively expressed the CRISPRi system. We cloned all the designed guide RNAs in a LV backbone and used them to transduce the previously mentioned cell lines.

Once identified, we generated two different sets of lentiviral (LV) plasmids for each regulatory sequence: (i) a control reporter plasmid for eGFP expression driven by the candidate sequences, and (ii) a therapeutic backbone for the expression of ITGB3 driven by the candidate sequences. These two types of plasmids allow easy tracking of expression levels of the reporter eGFP as well as the therapeutic *ITGB3* gene in a tissue-specific manner. Finally, we used these LVs to transduce *ITGB3*-deficient human MK cell lines to assess tissue-specific expression.

This approach enables us to conduct a comprehensive study of lineage-specific expression, which can be applied to develop new therapeutic approaches for GT and other IPDs.

P0542

An overview and comparative analysis of CRISPR activity prediction tools

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In silico design of CRISPR guide RNA (gRNA) with high efficiency and specificity is vital for successful application of the CRISPR-based gene editing technology. Although a large number of machine and deep learning (MDL)-based tools have been developed to predict the editing activity of gRNAs, a systematic and unbiased evaluation of their predictive performance and application scenarios is still needed. In this review, we provide a brief overview of the in-silico tools for CRISPR design and assess the CRISPR datasets and statistical metrics used for evaluating model performance. We benchmark six representative CRISPR/Cas9 editing efficiency prediction tools across six CRISPR datasets covering five cell types and two species. The deep-learning model CRISPRon outperformed other models, exhibiting more accurate and better performance. We compile all CRISPR datasets and in-silico prediction tools into a GUIDEnet resource web portal (https://dream.au.dk/tools-and-resources/guidenet), aiming to facilitate and streamline CRISPR design. Furthermore, we summarize features affecting CRISPR gene editing activity, providing importance insights into the model performance and the further development of more accurate CRISPR prediction models.

P0543

Enhancing homology-directed gene editing in hematopoietic stem and progenitor cells

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In the field of gene therapy, haematopoietic stem and progenitor cells (HSPCs) continue to be the ideal target cells for gene manipulation due to their long-term repopulation potential. Among the different gene manipulation strategies such as non-homologous end joining(NHEJ)-mediated gene editing, base editing, and prime editing, only the homology-directed repair(HDR)-mediated gene editing provides the option of inserting a large transgene under its endogenous promoter or any desired locus. Besides, HDR-mediated gene editing can be applied for the correction of point mutations and introduction of desired specific mutations. However, the poor efficiency of HDR is one of many challenges in exploiting the HDR pathway for HSPC gene therapy. In this work, we screened small molecules to identify the compounds with HDR-enhancing effect and identified a compound that improves HDR efficiency in the HSPCs. We extensively characterised the effect

of small molecule on the HSPCs and demonstrated high HDR efficiency at the gamma globin promoter both *in vitro* and *in vivo*.

P0544

Human ARMMs as a highly customizable non-viral platform for targeted delivery of therapeutic payloads

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A large proportion of therapeutic targets is confined to the intracellular compartment limiting treatment options with current biologic modalities. Developing strategies to efficiently deliver therapeutic payloads, such as protein, RNA or genome editors inside target cells or tissues would unlock a wide range of therapeutic applications. Major limitations of existing delivery platforms include achieving safe, precise, and efficient transfer of functionally active therapeutic cargos to specific cell types, through various administration routes. We have developed human ARMMs (arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles) as a highly modular, targeted non-viral therapeutic platform. In this study, we demonstrate that ARMMs can be engineered to present "homing" molecules that enable cell-specific delivery. We show that, by introducing cell-type specific surface engagers, ARMMs can be programmed to selectively target cell types of interest in mixed populations *in vitro*, ex *vivo*, and *in vivo*. We also demonstrate targeted functional delivery with gene editing complexes/mRNA to specific primary cells, as well as robust de-targeting of cells that do not possess the target receptors. Our data suggest the potential therapeutic utility of engineered ARMMs as a programmable human cell-derived non-viral platform for delivery of molecular cargo to specific cell types for a variety of gene therapy indications.

P0545

Potency and off-target evaluation of DMD base editing medicine *in vitro*

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DMD is a progressive muscle-wasting disease caused by mutations in the gene encoding the dystrophin protein. Exon 50 skipping can target 4% of DMD patients with large deletions. Until now, there is no drug for those patients. GEN6050 is an intravenously administered DMD exon 50 skipping base editing drug. The exon 50 skipping mechanism of GEN6050 is to modify the IVS50 5'SS in the human DMD gene and result in exon 50 skipping at mRNA level. For exon 50 skipping amenable DMD patients, exon 50 skipping generate a truncated yet in-framed dystrophin protein. Normal/DMD iPSCs derived myotubes/cardiomyocytes were treated with GEN6050 for 24hrs. After 7 days drug treatment, the cells were collected for off-target

evaluation or efficacy study. Untreated samples were used as control. The editing window of GEN6050 was defined as 10 bp upstream to 30 bp downstream of hE50-sgRNA (from +10 to -30 of hE50-sgRNA) corresponding to -24 to +36 bp of dystrophin gene IVS50 5'SS according to the efficient editing of GEN6050 (MOI=3E5) in normal iPSCs derived myotubes. Dose dependent target site editing, and exon 50 skipping were observed. The exon skipping efficiency is highly correlated with the editing efficiency, confirmed that the exon skipping is caused by efficient DNA modification at IVS50 5'SS. Similarly, dose dependent transgene transcription, target site editing, exon 50 skipping and dystrophin protein restoration were observed in both iPSC-DMD-del51-53 (exon 50 skipping amenable) derived cardiomyocytes and myotubes. High exon skipping were observed at the MOI of 1E6 in iPSC-DMD-del51-53 derived cardiomyocytes (80%) or myotubes (90%). Consistent with high exon skipping efficiency, up to 40% (MOI=1E6) dystrophin protein immunofluorescence restoration were observed in both iPSC-DMD-del51-53 derived cardiomyocytes and myotubes, indicating that GEN6050 has the therapeutical potential for exon 50 skipping amenable DMD patients.

Comprehensive off-target assessment was performed for GEN6050 in two different cell contexts, iPSCs differentiated myotubes and cardiomyocytes. For sgRNA-dependent off-target, we identified 36 potential sites by Cas-Offinder with mismatch <=3 or <=2 plus 1 DNA/RNA bulge. The top9 sites were deep sequenced, no potential sites were verified. For sgRNA-independent off-target, whole genome sequencing was performed (450X), with more than 30% on-target editing efficiency using non-treated sample as reference, comparable potential SNVs and indels were identified in GEN6050 non-treated control and GEN6050 treated samples. Using non-treated control as reference, 433 SNVs were identified in total, most of them (56%) are within intergenic region. No transcriptome level RNA off-target were identified.

In summary, GEN6050 leverages base editing activities to restore the Dystrophin protein in DMD iPSC derived cardiomyocytes/myotubes with low to non-detectable off-target risk. Our results indicate that GEN6050 can provide a potential cure for DMD exon 50 skipping amenable patients.

P0546

Expanding Base Editor Scope with Embedding Strategy

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Base editors can achieve base replacement without DNA double-strand breaks and have shown substantial potential in scientific research and gene therapy. Nevertheless, a variety of applications necessitate a range of unique features for base editors. For site-specific mutations, the base editor with narrow editing window is favored due to possible bystander mutations. In comparison, modifying the activity of regulatory elements on genome (e.g transcription enhancer), which is difficult to determine the exact regulatory sequences, requires a large editing window. To date, the editing windows of the majority base editors are +4 to +13 positions (+1 position at the far PAM end), which makes it difficult for effectively mutate these sites. Therefore, expanding the editing window will be another evolutionary direction. In this study, we evolve the Targeted AID-mediated Mutagenesis (TAM) cytosine base editor to expand its editing window using transposon technology. TAM CBE is a pioneer developed cytosine base editor that uniquely deaminate the ssDNA substrate, with an editing window of +1 to +12. First, we randomly inserted an activation-induced cytidine deaminase (AID) into the nSaCas9-KKH by MuA transposase to obtain a chimeric cytosine base editor (EB-TAM). We then introduced the EB-TAM library into E. coli along with a screening vector with inactivated kanamycin resistance. After IPTG induction, clones

grown on kanamycin plates indicated that functionally EB-TAM restored the antibiotic resistance. The insertion sites of AID were mapped by Sanger sequencing. We successfully obtained 11 EB-TAMs in the prokaryotic screening. Then, the editing efficiency and their editing windows of 11 EB-TAMs were identified by five C-rich endogenous sites in HEK293T cells. Among the 6 functional EB-TAMs identified in HEK293T, EB-TAM-007 showed modest to higher editing efficiency from +1 to +17 compared to the other 5 EB-TAMs. Since nSaCas9 (D10A)-KKH cleave the middle of the +17 and +18 nucleotides of the sgRNA targeting strand, it is theoretically difficult to develop a base editor to edit the +18, +19 and +20 nucleotide of the non-targeting strand. Therefore, we assume that EB-TAM-007 harbor the largest window among the reported cytosine base editors based on the nSaCas9 (D10A) KKH. To further improve the editing efficiency, the linkers between AID and SaCas9 in EB-TAM-007 were optimized with three different linkers (L1, L2, L3). The EB-TAM-007-L1 exhibited an enhanced editing efficiency, showing a 1.51-fold to 1.82-fold increase compared to EB-TAM-007-L3 and 1.75-fold to 3.75-fold increase compared to prototype TAM CBE. Finally, we employed EB-TAM-007-L1 to edit the DMD E53 splicing site. As expected, 20% editing efficiency and 50% E53 exon skipping were obtained in DMD iPSC-derived myotubes. In summary, we developed a novel CBE with high editing efficiency and large editing windows enable for effective editing, especially for regulatory elements.

P0547

Optimization of *ex vivo* gene editing protocol(s) in human hematopoietic stem cells (CD34+)

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For rare, monogenic disorders, gene therapy offers a promising therapeutic option in the absence of conventional therapies. *Ex vivo* manipulation of patient's own hematopoietic stem cells (HSCs) followed by transplantation allows the reconstitution of a healthy haematopoiesis. Genome editing can offer precise manipulation of cellular DNA sequences to modify the cellular traits/functions. CRISPR/Cas gene editing technology, along with an adeno-associated virus (AAV) as a DNA template, enables correction of errors in mutant genes to rescue disease phenotypes. A CRISPR/Cas ribonucleoprotein (RNP) induces double strand breaks (DSB) thereby activating DNA repair mechanisms: the homologous DNA recombination (HDR), and non-Homologous End Joining (NHEJ). HDR involves genetic exchange between the donor and the cellular DNA, is more precise, but also rare (active in S and late G2 phase) compared to NHEJ, that may create INDELS and is active throughout the cell cycle.

Gene editing protocols were established *ex vivo* in human HSCs, in the context of two different disease models, targeting two different genes. Human HSCs were pre-stimulated and then electroporated to receive an RNP (with the sgRNA specific for the locus of interest), together with an AAV delivering the donor DNA template encoding a GFP reporter protein and optionally a DNA-protein kinase (PKs) inhibitor to boost HDR. Addition of a DNA-protein kinase (DNA-PK) inhibitor led to a further increase in HDR efficiency. Similar results were obtained with the colony forming units (CFUs), showing equivalent editing outcomes. HDR efficiency results were also confirmed by droplet digital PCR (ddPCR).

The protocol was further optimized by introduction of a polymerase theta inhibitor which reduced unwanted genomic alterations. CAST-sequencing (single targeted linker-mediated PCR

sequencing) is planned to evaluate the benefit of polymerase theta inhibitors in preventing DNA INDELS.

In conclusion, we successfully established *ex vivo* gene editing protocols for human HSCs, applicable for two different genetic diseases. *In vivo* assessment of *ex vivo* edited cells will be addressed in NBSGW mice in terms of engraftment and normal haematopoiesis.

P0548

Base-editing mediated *MECP2* gene correction, a new gene therapy strategy for Rett Syndrome patients

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Gene-based approaches have exerted important therapeutic results in Rett syndrome (RTT) experimental models, raising great expectancy for soon achieving beneficial effects in patients. Many groups have in fact demonstrated that the restoration of *MECP2* expression could significantly ameliorate the pathological condition of preclinical models and in 2023 the first clinical trial started. However, one of the main concerns of this approach is the difficulty of regulating MeCP2 expression to recapitulate the endogenous condition. MeCP2 is differentially expressed in brain cells and its expression levels are crucial for cellular physiology.

To overcome this limitation, we decided to optimize an approach based on correcting the mutated endogenous *MECP2* in order to restore its expression under its own regulatory elements. Many of the most frequent RTT-associated mutations are in fact correctable by Adenine Base Editors (ABEs) technology. Therefore, to validate our approach, we considered the most frequently found mutation in patients, the *MECP2*-T158M. To date, we have screened and selected a sgRNA that allows us to revert the pathological mutation with an efficiency of over 30% in primary neurons derived from the *Mecp2*-T158 mutant mouse. Preliminary data have also shown good correction efficiency directly *in vivo* in the brain parenchyma of these animals, showing a Mecp2 restoration that mimic the physiological expression.

Although this approach is still in its early stages, it offers a potential future alternative to classical RTT gene replacement therapy, providing the advantage of greater safety for patients.

P0549

Therapeutic exon skipping through cytidine base editing in DMD mice model

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Loss of dystrophin protein causes Duchenne muscular dystrophy (DMD), characterized by progressive degeneration of cardiac and skeletal muscles. GEN60mE4 is a mouse DMD exon 4

skipping base editing product containing two AAV9 vectors, ss.AAV9.oTAM (optimized Targeted AID-mediated Mutagenesis) and ss.AAV9.mE4-sgRNA. GEN60mE4 target the IVS4 5'SS in the mouse Dmd gene, leading to interruption of the recognition of exon 4 during splicing process, resulted in exon 4 skipping. *Dmd*^{E4*} is a DMD mouse model has 4-bp deletion leading to dystrophin protein disruption. GEN60mE4 induced exon 4 skipping resulted in truncated yet in-framed dystrophin protein. PND21 *Dmd*^{E4*} /BL10 mice received a single IV administration of formulation buffer or different dose of GEN60mE4. Age-matched WT mice were included as controls. Interim and terminal sacrifices occurred at the end of 4-, 8-, and 28-weeks post-dose, respectively. The transcripts of oTAM were highly distributed in heart and skeletal muscles confirmed strong muscle specificity of SYN promoter. The sgRNA has been reported to be stabilized by forming a complex with Cas9 protein, similar expression pattern of mE4-sgRNA as oTAM was observed. Dose-dependent exon 4 skipping was observed in both heart and other tested muscular tissues. Especially in heart of 3E14 vg/kg GEN60mE4 group, exon skipping efficiency is close to 80%, the edited Dmd mRNA level was comparable to that of WT controls. The dystrophin protein reached approximately 20% or 7 % of the WT level in the heart or TA of the animals in 3E14 vg/kg group, respectively. Serum CK-MB exhibited a 2-fold decrease in 3E14 vg/kg group compared to untreated *Dmd*^{E4*} mice (P=0.0181). Deep RNA sequencing of heart tissue was performed to analyze the gene expression after treatment. The results showed that the genes that were differentially expressed in WT versus *Dmd*^{E4*} groups were highly rescued with GEN60mE4 treatment. Most importantly, TAM-based cytosine base editor induced non-detectable RNA off-target at the transcriptome level. Vector copies of oTAM and sgRNA gradually decreased over time over 28-week observation. Interestingly, the restored dystrophin protein dramatically decreased at 8-week and further catching up over 28-week in heart. The Dystrophin protein restored to 25.58% and 35.59% of WT level in 5E13 and 1E14vg/kg group at 28-week, respectively. Consistently, the editing efficiency decreased at 8-week in the heart of treated mice. In comparison, no decreased editing was observed in TA. The minimal editing efficiency in TA was observed (3%) at 4-week. At week 28, the editing efficiency accumulated to 12.4% in 5E13 or 11.28% in 1E14vg/kg treated mice. Immune response against transgene or AAV was evaluated at 8- and 28-week. Strong humoral and cellular immune response against AAV9 or cas9 but not dystrophin protein was observed, which might be a reason for the rapid reduction of editing efficiency at week 8 since the successfully transduced cells were easy to be attacked by immune cells. These studies demonstrate that TAM BE can restore the dystrophin protein and provide some therapeutic potential with long term observation. Immune response against AAV and transgene should be considered before human therapeutic application of base editors.

P0550

CRISPR/Cas9 system to correct laminopathic mutations in patients' cells: generation of *in vitro* models to identify biomarkers for Emery-Dreifuss Muscular Dystrophy and unravel pathogenic pathways in Hutchinson-Gilford Progeria

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Genetic mutations occurring in the *LMNA* gene and genes coding for nuclear envelope proteins, such as emerin (*EMD* gene) and BAF (barrier-to-autointegration, *BANF1* gene), give rise to a variety of genetic disorders collectively called laminopathies. To date, at least 11 distinct disease phenotypes can be defined within the laminopathy group. Among this heterogenous group, we are investigating type 1 Emery-Dreifuss Muscular Dystrophy (EDMD1) caused by mutations in *EMD* gene, and the type A Mandibuloacral Dysplasia (MADA) caused by mutations in *LMNA* gene and Hutchinson-Gilford progeria syndrome (HGPS) caused by an aberrant splicing in *LMNA* gene and characterized by the presence of premature ageing symptoms as MADA.

EDMD1 is a rare genetic X-linked disease caused by the absence of the emerin protein. A cure for this disease is not available to date and the identification of biomarkers for the evaluation of disease progression is mandatory. To accomplish this aim, we genetically corrected patients' myoblasts carrying a point mutation in the exon 1 of *EMD* gene, which abolishes the start codon, or a 5-nt duplication in the exon 6 of the gene leading to a frameshift and generating a truncated protein missing the transmembrane domain.

The CBE system was exploited to correct the exon 1 in myoblasts from patient #1 and the CRISPR/Cas9 nuclease, combined to a couple of gRNAs, was used to re-establish the correct frame, shifted by the duplication in exon 6, in myoblasts from patient #2. Gene-corrected patients' myoblasts were used as isogenic controls to identify four muscle-specific micro-RNAs altered in patients' cell cultures and rescued in CRISPR-corrected cells.

Hutchinson-Gilford progeria syndrome (HGPS) is one of the most severe disorders among laminopathies. HGPS is characterized by the presence of ageing-associated symptoms, including lack of subcutaneous fat, alopecia, growth retardation, joint contractures, osteoporosis, cardiovascular pathology, and death due to heart attacks and strokes in childhood. Many of these symptoms are in common to MADA, a rare disease with lipodystrophy and accelerated ageing, and to Mandibular Hypoplasia Deafness Progeroid Features and Lipodystrophy syndrome (MDPL) caused by *de novo* variants in *POLD1* gene.

HGPS, MADA and MDPL share clinical aspects as growth impairment with accelerated ageing, lipodystrophy, skin abnormalities and mandibular hypoplasia suggesting common stress-related pathogenetic pathways involved in premature ageing.

HGPS is caused by a single mutation in the exon 11 of *LMNA* gene. The mutation c.1824C > T results in activation of the cryptic donor splice site, which leads to the synthesis of progerin protein that accumulates at the nuclear envelope causing the described phenotype. To correct this point mutation in patient's myoblasts, we exploited the SpRYHF-ABE system. Preliminary experiments showed high frequency of the desired A to G conversion and detectable bystander effect, not associated to any known pathogenic variant. In conclusion, CRISPR-corrected cells from EDMD1 patients allowed the identification of micro-RNA signature as potential biomarkers of muscular dystrophy. CRISPR correction of HGPS, MADA and MDPL patients' cells will be instrumental to identify therapeutic targets for premature ageing diseases and ageing-related adipose tissue deterioration.

P0551

Enhancing Precision and Safety in CRISPR/Cas Gene Editing: A Combined Off-Target Detection Strategy

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The advent of CRISPR/Cas systems has revolutionized gene editing, providing unprecedented precision for modifying genomes to treat genetic disorders and advance biological research. However, the potential for unintended genetic alterations, or off-target effects, poses significant safety concerns. Current detection methods lack systematic approaches, underscoring the need for an integrated strategy that enhances safety protocols in gene editing applications.

Our presentation introduces a novel off-target detection approach, beginning with in silico predictions based on sgRNA sequences using multiple bioinformatics tools. This predictive foundation guides focused experimental validation. We employ a dual-technique strategy: iGUIDE-seq integrates unique molecular identifiers (UMIs) with sgRNA for precise tracking and amplification of genomic alterations, while Ligation target amplication PCR (LTA-PCR) amplifies extensive regions around predicted off-target sites, enhancing our detection capabilities.

From these methodologies, we compile a panel of potential off-target loci, rigorously tested against real samples to confirm our approach's effectiveness and practicality. By synthesizing computational predictions with empirical data, our strategy offers a comprehensive assessment of off-target effects, bolstering the safety and precision of CRISPR/Cas gene editing.

This integrated detection system represents a significant advance in gene editing technology. It provides a systematic, validated method for identifying and evaluating off-target events, ensuring safer and more effective gene therapies. Our approach promises to enhance the reliability of CRISPR applications, making it an essential tool for advancing precision medicine.

P0552

CRISPR/Cas9-mediated TCR replacement to target MAGE-A1+ metastatic melanoma cells

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Adoptive therapy with T cells engineered to express T cell receptors (TCRs) recognizing HLA-presented cancer antigens represents one the most promising strategy of precise therapy against solid tumors. Melanoma associated antigen 1 (MAGE-A1) is a member of the MAGE-A family of cancer/testis antigens, whose members are known to be highly expressed in multiple different tumor types. Of note, expression of MAGE-A1 antigens in normal tissue is limited to the immunopriviledge testis, which makes MAGE-A1 an ideal target for TCR-based adoptive cell therapy. Ongoing clinical trials with TCR-T cells, generated by retro- or lentiviral transduction, are

providing promising results against solid tumors, including non-small cell lung cancers and melanoma. Recently, the CRISPR/Cas9 system have been proposed to safely and effectively engineer T cells to redirect their antigen-specificity and enable them of enhanced anti-tumor response. In this study, we applied CRISPR/Cas9 system to replace endogenous TCRs with a cancer-specific TCR targeting MAGE-A1 in the context of HLA-A*0201, in T cells derived from healthy donors. Primary T cells were electroporated with ribonucleoproteins (RNPs) of Alt-R SpCas9 in complex to *TRAC* exon1-specific gRNA, and HDR donor template carrying MAGE-A1 TCR sequence, to trigger the knock-in in the *TRAC* locus. Moreover, simultaneous knockout of the endogenous beta chains was carried out with Alt-R RNPs targeting *TRBC* loci. Genomic and cytofluorimetric analyses showed almost complete knock-out of endogenous TCR chains, and more than 14% CD8 T cells expressing the MAGE-A1-specific TCR upon precise HDR events, representing 85% of the TCR+ cells in the CD8 compartments. Genotoxicity of the CRISPR-treatment was evaluated to detect chromosomal rearrangements between the target loci by ddPCR, as well as induction of p53 pathway. Engineered T cells were sorted, expanded and analyzed for their ability to recognize and respond to MAGE-A1-positive cancer cells. We selected six primary metastatic melanoma cell lines expressing HLA-A*0201 and different levels of MAGE-A1 transcript. Co-culture experiments of TCR-redirected T cells with target cells demonstrated that edited T cells strongly activate upon recognition of targeted cells by producing proinflammatory cytokines, such as inteleukin-2 (IL-2), interferon-γ (IFNγ), and tumor necrosis factor(TNF). To measure the killing activity, T cells expressing MAGE-A1-specific TCR were co-cultured with target cells previously transduced with a lentiviral vector carrying an expression cassette for GFP to induce the expression of the reporter gene in the target cells. Flow cytometric analysis of the residual GFP+ target population in co-culture experiments showed that engineered T cells were able to kill metastatic melanoma cells. In conclusion, these data encourage the application of CRISPR-mediated non-viral TCR editing to generate highly efficient T cells able to kill MAGE-A1-expressing cancer cells.

P0553

Unravelling pathogenetic mechanisms of autosomal dominant retinitis pigmentosa triggered by C-terminal mutations in rhodopsin gene

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Rhodopsin (RHO) mutations represent one of the most common causes of retinitis pigmentosa (RP), accounting for 30-40% of autosomal dominant RP (adRP) and 8 to 10% of all RP. More than 200 RHO mutations have been documented in humans leading to RP (https://www.hgmd.cf.ac.uk/ac).

Although the disease-causing gene is known and the P347S and P347L C-terminal RHO variants, the most recurrent RHO pathogenic variants in Europe, are strongly associated with a severe adRP phenotype, the pathogenic mechanisms underlying retinal degeneration and cell death are not clearly elucidated.

Pathogenic variants in the C-terminal tail of the protein (P347S, P347L, P347R, V345M, Q344ter and S334ter) disrupt an essential motif for rhodopsin to be correctly transported to outer segments (OS). Various mechanisms that lead to the induction of apoptosis have been proposed

for the C-terminal RHO variants. For example, the metabolic burden caused by the continuous degradation of the mis-trafficked RHO, the induction of the unfolded protein response, a mitochondrial dysfunction and fragmentation and the interference of RHO variants with cellular processes including intracellular signalling could contribute to trigger pathogenetic mechanisms of retina degeneration.

Human retinal cells from foetal retina, post-mortem explants and iPSC based retinal organoids would provide excellent resources for studying disease mechanisms. However, all these sources of retinal cells are difficult to obtain and display variability that might impact transcriptomic analysis, and require a labor intensive and expensive culture procedures that limit the research. Human immortalized RPE1 (hTERT-RPE1) cells represent the *in vitro* human model commonly used to induce ciliogenesis, and contain abundant mitochondria to deal with metabolic demand, similar to photoreceptors. In this study, we used hTERT-RPE1 cells to investigate cilium organization and trafficking of rhodopsin carrying P347S and P347L mutations.

To ameliorate and favour the traffic of WT RHO to the cilium we generated a RHO-EGFP-C8 fusion protein which successfully localized in the ciliary extension of hTERT-RPE1 cells. The P347S and P347L variants were introduced in the WT fusion protein, allowing the evaluation of trafficking defects by confocal analysis. We are evaluating mitochondrial dysfunction by quantification of oxygen consumption rate (OCR) and cytotoxicity by LDH assay. To assess if and to what extent the correction of the toxic variants will rescue the observed defects *in vitro*, we are employing CRISPR ABE system delivered as mRNA or VLP into hTERTRPE1 cell. Side by side comparison of human RPE1 cells expressing the toxic RHO variants, WT RHO or ABE-corrected RHO will provide insights into the molecular mechanisms leading to adRP and might prompt pathogenetic studies in more relevant cells model as human retinal organoids.

P0554

Oral MSCs -Derived Exosomes Loaded with hBMP-2: A Path to Personalized Bone Regeneration

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Introduction

Traditional bone regeneration methods have long relied on bone substitutes and biomaterial scaffolds to facilitate the intricate process of new bone formation. This process involves a series of orchestrated events, including progenitor cell recruitment, angiogenesis, and dynamic microenvironment changes. Currently mesenchymal stem cells (MSCs), with their self-renewal and multi-differentiation potential are used in regenerative therapies. The latest research on MSCs has highlighted their ability to differentiate into adipogenic, chondrogenic, osteogenic, endothelial, neural, and epithelial lineages both in vivo and in vitro.

MSCs have been found to produce growth factors and other bioactive molecules stored in exosomes, which are small particles surrounded by a bilayer-lipid membrane. These exosomes

contain specific proteins and RNA that facilitate cellular and tissue regeneration and proliferation. Isolated MSCs-exosomes have shown properties similar to their parent cells and have been successfully utilized in research settings.

This study aims to pioneer a novel bone regeneration strategy by harnessing the synergistic potential of (hMSCs genetically modified with lentiviral vectors expressing bone morphogenetic protein 2 (BMP-2), in conjunction with their therapeutic exosomes derived from hMSCs-BMP-2.

Materials and Methods

MSCs were isolated from patients requiring implants. The cells were tested by flow cytometry for positive MSCs-associated surface markers (CD73, CD90 and CD105) and negative markers (CD34 and CD45). These cells were transduced with a lentiviral vector containing BMP-2 and BMP-2-GFP, for the overexpression of BMP2 and GFP. Cells were cultured in conditioned medium (osteogenic differentiation medium) and compared with cells cultured in non-conditioned medium. RNA extraction was performed at 7-, 15-, 21-, and 28-days post-transduction, followed by reverse transcription to obtain cDNA. Quantitative PCR (qPCR) was carried out to measure the expression of BMP2, OCN, ALP, and RUNX2. Western blot analysis was performed to determine the BMP2 protein levels in different conditions. ELISA was used to determine the BMP2 protein levels in the cell culture supernatants at various time points.

Results

Flow cytometry analysis confirmed the expression of predicted markers, with CD73, CD90, and CD105 being positive and CD34 and CD45 being negative throughout the 28-day period. Additionally, differentiation into three lineages (osteogenic, adipogenic, and chondrogenic) was confirmed through staining with Alizarin Red, Oil Red, and Alcian Blue. The ELISA and Western blot assays demonstrated the overexpression of BMP2 by the transduced cells. The qPCR assays also show that MSCs-BMP-2 cells have a higher expression of osteogenesis genes (RUNX2, ALP) compared to WT cells, with this expression being even greater when the cells were cultured in conditioned medium.

Conclusions

We succesSfully genetically modified hMSCs to overexpress BMP-2; CD9 and elevated BMP-2 expression induced relevant genes associated with osteogenic differentiation and BMP-2 is encapsulated within EVs, which enables protein exchange between cells.

P0555

Genetic engineering opportunities for cultivated meat cell line development

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Cultivated meat (also referred to as 'cultured' or 'cell-based' meat) has emerged as a promising innovation to address modern challenges in food production, distribution, and security. However, none of the cell types or cell lines currently available in the industry possess the full array of phenotypes necessary for cost-effective, industrial production. In addition to immortalisation, other phenotypes such as rapid proliferation in low-cost culture medium, viability in high-density culture, metabolic efficiency, waste metabolite tolerance, shear stress resistance, genetic stability

and numerous others will also be necessary to enable production at the cost and scale needed to compete with commodity products. Here, I will discuss a variety of phenotypes and the spectrum of gene editing strategies that might be used to engineer them into mammalian cell lines. I will also discuss regulatory attitudes towards these different techniques, and argue that careful selection of tools can allow powerful cell line engineering whilst mitigating the majority of regulatory and consumer acceptance concerns.

P0556

Engineering CRISPR nucleases for allele-specific editing of dominant genetic diseases

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Ectrodactyly-Ectodermal dysplasia-Cleft (EEC) syndrome is an autosomal dominant genetic disease caused by point mutations in the TP63 gene, characterized by a triad of ectrodactyly, ectodermal dysplasia, and facial clefting, along with several other clinical manifestations such as corneal blindness. Although surgery can improve some aspects of the patient's life, the biggest unmet medical need is blindness caused by corneal opacification as a result of the progressive deficiency of limbal stem cells.

We identified three EEC syndrome-causing mutations that form a common 5'-TATG-3' PAM and identified Cas12a-RVR as a potential candidate to be improved for targeting these genetic alterations. The 5'-TATV-3' PAM recognized by Cas12a-RVR is too broad to ensure high specificity of the nuclease. Therefore, we rationally engineered CRISPR/Cas12a (formerly Cpf1) to target mutations that cause EEC syndrome by identifying the amino acid residues that interact with the PAM and substituted them with other residues that form a more specific bond with the desired 5'-TATG-3' bases. We conducted energy fold change analyses to ensure that the structure remains stable and increases specificity towards the desired PAM. We selected two amino acids as the best candidates and inserted these modifications into plasmids carrying the sequence for Cpf1-RVR, either as a single or in combination.

Engineer CRISPR/Cas12a variants showed a high editing efficiency on the mutated target gene and a low off-target activity on the WT sequence. Since EEC syndrome is sporadic, we validated our engineered CRISPR system in a biallelic EEC syndrome cellular model. This cellular system enables us to rapidly evaluate through fluorescence the gene editing efficiency on both the mutated and the wild-type allele.

Overall, this rational approach opens the door for the potential development of more engineered Cas proteins to target almost any dominant mutation.

P0557

CRISPR/Cas9 high-throughput screening and validation of epigenetic modulators in Ewing sarcoma

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Ewing's sarcoma (ES) is a highly aggressive and rare tumor primarily affecting the bones and soft tissues of children and adolescents. This malignancy is characterized by the presence of fusion oncogenes, typically *EWSR1::FLI1*, arising from the chromosomal translocation t(11;22)(q24;q12). Approximately one-third of patients experience relapses and metastasis, with a 5-year survival rate of around 20%, which highlights the urgent need for new, specific and effective treatments. In this sense, novel strategies targeting the cancer epigenome are being currently explored. In fact, epigenetic modifications play a crucial role in the development of ES, since the *EWSR1::FLI1* not only acts as a transcriptional modulator of essential genes, but also induces epigenetic modifications of the transcriptome. Therefore, we hypothesized that high-throughput CRISPR/Cas9 screening of epigenetic modulators may allow to identify potential candidates essential for ES cells survival and proliferation. By employing a negative selection screening and following the MAGeCKFlute gold-standard analysis pipeline, we were able to identify a set of ten potential candidate genes. These candidates were filtered by assessing their genetic dependency across different ES cell lines and evaluating the effect on ES cell proliferation when inhibiting their expression. Finally, the candidate *MARCHF5* was selected for *in vitro* validation. Knocking out *MARCHF5* in ES cells resulted in decreased cell viability and increased apoptosis, as confirmed by cell count and annexin V analysis. Besides, RNA sequencing of *MARCHF5* knockout cells revealed an altered gene expression profile, with significantly expressed genes involved in energy production and lipid metabolism. Further analysis is necessary to confirm these preliminary results and to determine the role of this epigenetic modulator in ES cells. The ultimate goal of this project is to translate these findings into the identification of a new potential therapeutic target, which could be leveraged to improve the existing therapies for ES patients.

P0558

An NGS method for qualifying sequence impurities in therapeutic CRISPR sgRNAs

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Advances in the manufacture of chemically synthesized single-guide RNAs (sgRNAs) have made CRISPR a promising technology for use in gene therapy. As a clinical product, evaluating sgRNA reagent purity is of great importance for patient safety. Traditionally, analytical chemistry methods such as HPLC (high performance liquid chromatography) have been used to interrogate the purity of these chemically synthesized oligos; however, these methods do not provide any information about the sgRNA sequence. Due to process impurities, the presence of alternative

sgRNA species in the sample could lead to deleterious patient effects. NGS (next-generation sequencing) technology has reshaped diagnostic testing and made in-roads in qualifying the purity of other clinical grade RNA products such as mRNA vaccines. Here, we describe an NGS-based method that provides sequence level information of sgRNA samples, including the detection of low level alternative sgRNA species. Extensive optimization has been performed to reduce background analytical artifacts that can confound signal. The method we describe can detect an alternative sgRNA species at a limit of 0.1% in a background of wild-type guide. This method can be used as an orthogonal purity metric to traditional HPLC while providing additional critical information regarding sgRNA sequence.

P0559

Development of Erythroid-Specific Cellular Models for Beta-Thalassaemia Using Base Editing Technology

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Beta-thalassaemia is a prevalent monogenic disorder caused by mutations in the β-globin gene (HBB). These mutations lead to reduced or absent synthesis of the β-globin chain, which normally combines with two α-globin chains to form the haemoglobin tetramer. The excess of free α-globin chains results in severe red blood cell dysfunctions, ineffective erythropoiesis, anaemia, and impaired oxygen delivery. Base editing (BE) is an innovative technology enabling efficient single-nucleotide conversion at specific target sites without causing DNA double-strand breaks. The paucity of cellular models harbouring the exact mutations responsible for beta-thalassaemia limits the application of BE. Our aim is to establish an erythroid-specific cellular model harbouring beta-thalassaemia-causing mutations using BE. We selected mutations in the promoter, initiation codon, intronic, and exon regions of the HBB gene known to affect various stages of β-globin production. BE strategy was initially tested on the K562 cell line, resulting in variable but efficient HBB targeting with minimal bystander effects or targeting of the β-globin paralog (HBD). However, the K562 model is characterised by the presence of a large fraction of other globins, primarily γ- and ε-globin, expressed during human development, which limits the phenotypic effect of HBB gene mutation. Therefore, selected BE strategies were transposed to HUDEP-2, an erythroid cellular model with globin gene regulation resembling that of adult individuals. The delivery of BE systems in HUDEP-2 cells resulted in efficient targeting with no toxic effects or negative selection signs. Erythroid differentiation revealed a reduced erythropoiesis ability in mutation-harbouring cells compared to untreated ones. Additionally, apoptosis analysis showed higher mortality in treated cells. qRT-PCR data indicated that β-globin expression decreased following BE efficiency and predicted severity of the mutations. HPLC analysis confirmed that protein expression levels were reduced due to the decreased beta-globin expression as expected. We have established a cohort of mutation-specific cellular models with the dual purpose of gaining new insights into the cellular mechanisms of beta-thalassaemia and testing therapeutic BE protocols for the reversion of beta-thalassaemia-causing mutations.

P0560

A mutation-independent RNA editing strategy based on RHO-targeting trans-splicing ribozyme for treatment of retinitis pigmentosa

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Retinitis Pigmentosa (RP) is the most prevalent hereditary degenerative eye disease, characterized by abnormalities in the photoreceptors (rods and cones) or the retinal pigment epithelium (RPE) of the retina. Rhodopsin mutations account for about 25~30% of autosomal dominant retinitis pigmentosa (adRP). Although the Pro23His (P23H) mutation in the rhodopsin (RHO) gene is the most common cause of adRP in North Americans, over 150 types of autosomal dominant mutations have been identified in the *RHO* gene. The Group I intron-based trans-splicing ribozyme enables the reprogramming of target RNA into the gene of interest through RNA replacement. In this study, we developed a specific trans-splicing ribozyme as a mutation-independent therapeutic strategy to replace endogenous RHO RNA with exogenous functional RHO RNA and used AAV system as a delivery vehicle. The most accessible target site of RHO RNA was identified by RNA mapping using a ribozyme library. Sequencing analysis of trans-splicing reaction sites revealed that the most efficient target site occurred at the uridine base (U) in the RHO RNA 5' UTR. Ribozymes targeting the 5' UTR have the potential to target all types of RHO mutations because all mutations are present in the open reading frame region of the RHO gene. To improve the trans-splicing specificity and efficiency of the ribozyme, we modified and optimized the structure of the ribozyme targeting the most accessible site of the target RHO RNA. The candidate with the highest trans-splicing efficiency was then selected through comparative in vitro analysis using cell lines. Importantly, we observed that mutant RHO RNAs were trans-spliced and restored to human wild-type (WT) RHO RNA with high fidelity and efficacy with negligible off-target effects by the specific RHO-targeting ribozyme in cells. Diverse mutation types of RHO RNA were successfully edited by the single RHO-specific ribozyme in cells. To verify in vivo function, an AAV vector encoding the optimal RHO-targeting ribozyme was constructed and delivered by subretinal injection into the eyes of P23H or Q344X human Rho (hRHO) knock-in (KI) mice at postnatal week 5. We evaluated rod-isolated retinal function of AAV encoding the ribozyme using the electroretinogram (ERG) and compared with untreated or PBS-treated control mice up to 26 weeks post-injection. The scotopic b-wave amplitude of eyes in the untreated or PBS-treated P23H or Q344X hRHO KI mice significantly decreased compared to wild-type mice. In contrast to controls, the b-wave amplitudes of the ribozyme-encoding AAV treated eyes showed significant and dose-dependent increases, which were maintained until 26 weeks. Molecular and cellular analysis of retina tissue showed that human P23H RNA was accurately replaced with human WT RHO, and the outer nuclear layer (ONL) appeared significantly thicker in the RHO-targeting ribozyme treated eyes. This study demonstrated that the ribozyme effectively prevents rod photoreceptor degeneration and preserves their function in both P23H or Q344X hRHO KI mice. These results suggest that RNA editing approach based on RHO-targeting ribozymes could be a potent and mutation-independent therapeutic strategy for RHO-adRP patients.

P0561

CRISPR/Cas9-mediated allele specific disruption of a dominant *TP63* mutation in human primary EEC derived keratinocytes

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Ectrodactyly-Ectodermal Dysplasia-Cleft lip (EEC) syndrome is a rare dominant genetic disease caused by *TP63* missense mutations in the DNA Binding Domain (DBD). EEC patients display skeletal phenotypes such as polysyndactyly and cleft lip palate. Moreover, certain mutations lead to the depletion of limbal stem cells, resulting in progressive blindness. While surgical interventions can address some skeletal defects, there is an urgent medical need to address corneal blindness, which significantly impacts the quality of life for affected individuals.

Here, we propose the development of an allele-specific gene editing strategy to correct R280C (C>T) *TP63* mutation, causative of EEC and linked with ocular defects. Due to the presence of an -NGG PAM sequence adjacent to the C>T mutation, we designed a guide RNA (gRNA) for SpCas9 to specifically target the mutated allele. The ribonucleoprotein (RNP) complex was electroporated in EEC patient-derived primary keratinocytes, as well as in healthy human primary keratinocytes as control. Since the only difference between the two alleles is represented by the R280C mutation, we considered genomic context of the healthy keratinocytes as the worst-case scenario for the off-target activity of the SpCas9. The editing efficiency was assessed by locus-specific amplification and sequencing, followed by editing analysis via the TIDE analysis tool. We observed low editing activity on normal human keratinocytes, while achieving a high-efficiency knockout of the mutant allele in EEC primary keratinocytes. This initial set of data demonstrated the ability of the designed SpCas9+gRNA complex to discriminate between the two alleles, editing preferentially and more efficiently the mutated one.

Moreover, considering the nature of p63 as a crucial transcription factor in the control of stem cell proliferative potential and differentiation process in keratinocytes, we functionally demonstrated that editing occurred only on the mutated allele. Specifically, we observed the rescue of expression for several p63 transcriptional targets. Strikingly, the editing efficiency evaluated at the genomic level was consistent with the functional rescue of transcriptional regulation of p63 targets after the editing process.

Potential activity at *in silico* predicted off-target site(s) was assessed by Sanger sequencing and showed no editing activity, therefore demonstrating a safe and specific editing process.

Collectively, these preliminary data show the feasibility of CRISPR/Cas9 technology to specifically knock-out the R280C *TP63* mutation and its potential for the restoring p63 transcriptional activity.

P0562

Identification and validation of factors promoting resistance to CAR-T therapy in Multiple Myeloma by genome-wide CRISPR screenings

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CAR-T cells have revolutionized cancer immunotherapy, representing a promising option for relapsed/refractory Multiple Myeloma (MM) patients. Despite high remission rates observed after BCMA CAR-T therapy, there is a lack of long-term responses since most of the patients relapse within the first year after treatment (media PFS 13.8 months for ide-cel and 34.9 months for cilta-cel). Development of primary, and especially, secondary resistance to CAR-T therapies, mainly due to antigen scape mechanisms, is still a relevant clinical problem. The main aim of this work was to identify new mechanisms of resistance to CAR-T therapies in MM beyond antigen loss.

We performed a genome wide CRISPR screening in MM cell lines using the CRISPR Brunello library, containing 76456 sgRNAs targeting 19114 coding genes. MM1S and KMS11 cell lines were engineered to express Cas9 and then transduced with Brunello library at MOI of 0.2 to ensure individual sgRNA transduction. MM1S and KMS11 cells were then cocultured with BCMA CAR-T cells at a E:T ratio of 1:25. We used MAGeCK algorithm, that evaluates the statistical significance of individual sgRNA abundance changes using a negative binomial model, to identify top sgRNA hits by comparing samples after 14 days of cocultured with initial controls. Cytotoxicity was evaluated by using Bright-Glo™ Luciferase Assay System. *In vivo* antitumoral efficacy was evaluated using xenograft MM models in NSG mice.

Genome-wide CRISPR screening allowed the identification of 5 targets (undisclosed) whose absence conferred resistance to BCMA CAR-T cells. Individual target validation was performed in both MM1S and KMS11 cells, generating at least 3 independent clonal cell lines depleted of each of the identified factors (MM^{KO} cells). Disruption of the corresponding target in each MM^{KO} cells was confirmed by WB. Additionally, we corroborated that target depletion did not affect neither BCMA expression nor cell growth capacity. Interestingly, coculture of the generated MM^{KO} cell lines with BCMA CAR-T cells showed that depletion of one of the 5 identified factors improved MM cell survival. Moreover, *in vivo* analysis in NSG mice showed a reduced antitumoral efficacy of BCMA CAR-T cells in animals injected with MM^{KO} cell depleted from that specific factor, validating their capacity to promote resistance to BCMA CAR-T cells. Additional transcriptional and functional analyses are being performed to fully characterize pathways involved in resistance induction after inhibition of that specific target.

Overall, our genome-wide CRISPR screening allowed the identification and validation of undisclosed targets whose inhibition provide resistance to CAR-T cell treatment in MM, representing a potential target for the development of improved CAR-T therapies.

P0563

Novel adenine base editor variants with single base selectivity to minimize bystander editing

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Base editing is a recently developed molecular tool that enables the conversion of one DNA base into another without DNA double-strand breaks or relying on donor templates. Adenine base editing (ABE) involves the fusion of a DNA-modifying deaminase (derived from E.coli TadA) with a catalytically inactive or nickase Cas9, which enables the conversion of A to I within a specific window of the non-target DNA strand. Despite its tremendous potential and its inherent safety compared to conventional gene-editing tools, such as homology directed repair, BE can also introduce undesired base changes, either due to bystander deamination, gRNA-mediated off-targets (OT) or non-gRNA-mediated OTs. Bystander off-targets are due to unwanted editing of nearby bases in close proximity of the target A within the editing window. These changes may result in amino-acid (AA) substitutions or disruption of regulatory sequences, severely limiting therapeutic applications of BE. While published advance-generation ABE are extremely efficient and compatible with a wide range of applications, safe clinical translation requires strict control of genomic off-targets effects. We recently described a novel approach to enable the use of adoptive immunotherapies for acute myeloid leukemia by precise epitope-editing of the target antigen (such as FLT3 and CD123) on donor hematopoietic stem cells (HSPCs). The target modification endows HSPCs with selective resistance to CAR-T or monoclonal antibodies, thus minimizing on-target/off-tumor toxicity and improving both safety and anti-leukemia efficacy. We hypothesized that, as epitope editing allows easy detection of BE outcomes by flow cytometry in human cells, we could exploit it for directed evolution of novel ABE with novel properties. To test this concept, we generated a Sleeping Beauty degenerated library constitutively expressing SpRY-ABE8e with random substitutions within E.coli TadA-8e, and introduced it into human reporter cell lines. By taking advantage of our epitope mutations, we interrogated the TadA library with FLT3- and CD123-targeting sgRNAs, among others, FACS-sorted edited cells and sequenced the library region by NGS. We identified several new TadA variants that, when re-tested individual, displayed drastically different window selectivity, with some achieving near-perfect on-target single base selectivity for FLT3 (up to 82.5% vs 32.4% for ABE8e for A6) and minimal bystander deamination (-74.4% A7 editing compared to ABE8e), while retaining overall efficiency (87.6% compared to ABE8e). In a similar way, for CD123, novel ABE variants showed superior control of by-stander activity, with complete elimination of A9 editing while retaining on target deamination of A6. We next validated these findings by preparing mRNA through in vitro transcription of 24 top candidates and re-tested them on CD34+ HSPCs. Critically, we were able to confirm the same base selectivity shown in cell line experiments also in primary HSPCs. We here highlight the possibility to develop novel, bespoke base editing tools with enhanced precision for the intended targets, broadening the range of therapeutic applications for human health and ensuring the safer translation of current gene therapy techniques.

P0564

Tales from R-loops: how RNA/DNA hybrids may influence AAV-mediated genomic integrations in mouse liver

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AAV vectors have a natural capacity to stimulate homologous recombination (AAV-HR), however they have recently drawn attention for exhibiting the propensity of random integrations across the genome. In our prior studies, we demonstrated the induction of homologous recombination in mouse liver utilizing a promoterless AAV vector flanked by host genomic homology arms containing a therapeutic coding sequence. Despite achieving therapeutic efficacy in select animal models, the frequency of edited cells remained suboptimal (~1.0%).

Thus, we conducted a genetic screen (deAlencastro et al., 2021) that led to the identification of Fanconi Anemia complementation group M (FANCM) as a key determinant influencing AAV-HR, which is also among the helicases crucial for resolving DNA/RNA hybrids, known as R-loops. In our study, inhibition of FANCM and SRSF1, enzymes involved in R-loop dissolution, significantly improved AAV-HR efficiency in vitro. Additionally, treatment with topotecan, a small molecule that increases genome-wide R-loop levels, enhanced AAV-HR in both a murine hepatoma cell line (HEPA1-6) and mouse liver in vivo. Mechanistically, this enhancement was corroborated by a decrease in AAV-HR efficiency upon co-treatment with RNase H1, which degrades the RNA strand of the DNA/RNA hybrids.

Furthermore, we utilized DNA/RNA immunoprecipitation sequencing (DRIP-seq) to conduct a comprehensive mapping and quantification of R-loop formations across the genome, representing the first genome-wide R-loops map of an intact tissue. Our findings unveil a pronounced enrichment of G-quadruplex-forming motifs at the identified mouse DRIP-peaks, suggesting a prevalent presence within gene promoters, where G-quadruplexes are known to exert regulatory functions. Moreover, a comparative analysis highlights elevated DRIP peaks in HEPA 1-6 cells relative to liver samples which potentially correlates with a higher transcriptional activity in transformed cells in contrast to quiescent hepatocytes within the liver.

Our observed variations in DRIP peak genomic location between HEPA 1-6 cells and murine liver potentially underscored the contextual role that R-loops possibly play based on whether a cell is transformed or quiescent. In addition, we observed increased levels of R-loop formation in the *Albumin* gene region (exons 12-14) both in HEPA 1-6 and mouse liver, coinciding with the regions where we designed homology targeting arms. Conversely, *Albumin* regions with lower or negligible R-loop levels showed minimal AAV-HR activity.

Beyond promoting enhanced AAV-HR, our findings suggest that genomic regions with high levels of R-loop formation may represent a genomic feature for "random" AAV integration. Recent studies have located integrated AAV vectors in the liver to genomic regions where we found high concentrations of R-loops. Experiments in mice constitutively expressing SpyCas9 are underway to characterize the influence that genomic R-loops might have on the efficacy of CRISPR/Cas9-mediated genome editing in murine liver.

These insights may foster studies for strategies to improve the efficacy of genome editing and enhance our ability to predict regions prone to insertional mutagenesis with therapeutic viral vectors.

P0565

Base editing-mediated correction of severe β^0 thalassemia mutations: efficacy and genotoxicity studies

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Beta-thalassemia is a recessive disease caused by mutations in the β-globin gene (*HBB*) locus which reduce (β$^+$) or abolish (β^0) the production of hemoglobin β-chains leading to a severe anemia. Sickle cell disease (SCD) is a recessive disorder due to a single amino acid change in the β-globin leading to hemoglobin polymerization, which causes red blood cell (RBC) sickling and hemolytic anemia.

Transplantation of autologous, genetically modified hematopoietic stem/progenitor cells (HSPCs) is an attractive therapeutic option and two gene therapy products have been recently approved by the FDA. However, current gene therapy strategies based on the use of lentiviral vectors or CRISPR/Cas9 nuclease are not equally effective in all patients and/or might raise safety concerns. Base editing (BE), a CRISPR/Cas9 derived genome editing tool, allows the introduction of precise point mutations within the genome. Here, we evaluate the efficacy and safety of BE-mediated mutation correction approaches.

CD39 (CAG>TAG) and IVS2-1 (G>A) β^0 mutations are among the most common and severe β-thalassemic mutations in the Mediterranean area and in Middle East. We designed two BE strategies allowing gene correction with efficiencies of up to ~90% in β-thalassemic HSPCs. This led to high β-globin levels in RBCs *in vitro* differentiated from edited HSPCs and the correction of the delayed erythroid differentiation.

In parallel, we tested our strategies in the context of SCD-β-thalassemia, a SCD subtype caused by the co-inheritance of the SCD mutation and a β-thalassemia mutation on the other allele. BE allowed the correction of the thalassemic *HBB* allele, leading to the decreased frequencies of apoptotic precursors and sickled RBCs.

To evaluate the safety profile of our approaches, we nominated off-target sites by *in silico* prediction and GUIDE-seq. Overall, we did not identify off-target mutations with anticipated clinical relevance. While the homologous *HBD* gene was detected as an off-target site in the CD39 mutation correction strategy, BE preserved the integrity of the β-globin locus as evaluated by long-read sequencing of the region encompassing these adjacent on- and off-target sites. Moreover, whole exome sequencing and RNA-seq of control and edited β-thalassemic HSPCs showed no discernible impact of the editing procedure on the DNA A>G mutational burden or on the transcriptome.

Finally, xenotransplantation experiments showed no impact of the BE strategy on the engraftment and differentiation potential of edited HSCs. We reached up to ~70% of gene correction efficiency in the total human cells engrafted in the bone marrow of transplanted mice and across the different lineages, showing no skewed hematopoiesis. Of note, off-targets were detected also *in vivo* with a kinetics that parallels that observed at the on-target site, showing no counter-selection of HSCs carrying off-targets events. Importantly, we detected high β-globin levels in bone marrow human erythroid edited cells and an increased frequency of circulating human RBCs, confirming the correction of the β-thalassemic phenotype *in vivo*.

Overall, our study demonstrates that gene correction using BE is safe and efficient in *bona fide* HSCs, thus paving the way for the clinical development of these approaches for the treatment of β-thalassemia and SCD-β-thalassemia.

P0566

From discovery to evolution of novel Cas orthologs: optimizing CoCas9 for gene therapy applications

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Despite the great success of CRISPR tools for genome editing applications, most currently characterized tools are not fully compatible with gene therapy applications due to delivery constraints and target range limitations. To leverage its full potential for gene therapy, many efforts are placed to retrieve novel orthologs from the natural reservoir, aiming at expanding the gene editing toolbox. Nonetheless, the majority of CRISPR-Cas variant systems that have been identified so far are poorly active in mammalian cells and are thus incompatible with gene therapy applications. To improve the activity of novel Cas orthologs molecular engineering is needed.

In this work, we have used a directed evolution platform developed in our laboratory, Eukaryotic Platform to Improve Cas Activity (EPICA), to evolve a novel compact Cas9 ortholog, CoCas. The EPICA platform is based in *Saccharomyces cerevisiae* model, a eukaryotic system that provides a nuclear context closer to the mammalian cells environment. By coupling the Cas nuclease activity with yeast auxotrophic selection based on the reconstitution of ADE2 and TRP1 genes, EPICA was key to enhance other ortholog, UltraCjCas9 (Ruta et al, Genome Biology 2024). In this work, we focused on improving the activity of CoCas9, a novel high-fidelity and compact (1004 amino acids) variant that was discovered by our group by massive interrogation of metagenomic data (Pedrazzoli et al, Nature Comm 2024). Using EPICA, we have been able to generate multiple mutations that altogether improve CoCas9 overall activity, making it a valuable candidate for gene therapy applications.

This study shows the power of our discovery-optimization pipeline to increase the variety of genome editing toolbox for the advancement of gene therapy.

P0567

Prime editing-mediated *COL17A1* repair in junctional epidermolysis bullosa

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Prime Editing (PE) is a sophisticated CRISPR/Cas9-based gene editing technology, which combines a beneficial edit to indel ratio with low off-target activity. A Cas 9 H840A nickase-reverse transcriptase fusion protein in combination with a 3'-extended sgRNA (pegRNA) allows for the precise correction of all types of point mutations, small insertions and small deletions without requiring double strand DNA breaks. Epidermolysis bullosa (EB), a monogenetic disorder associated with mutations in at least 16 genes, is characterized by extensive skin blistering upon minimal mechanical stress. The subtype junctional EB (JEB), defined by skin cleavage within the lamina lucida of the basement membrane, can be caused by mutations in *COL17A1*, which lead to loss of functional type XVII collagen (C17). In this patient-specific study, we used prime editing to correct two compound heterozygous *COL17A1* frameshift mutations (c.3569insG in exon 50, c.4000delA in exon 52) in patient-derived primary JEB keratinocytes to restore C17 expression.

For this purpose, prime editor mRNA encoding the PEmax fusion protein was transcribed *in vitro*. Primary JEB keratinocytes were subsequently transfected with the prime editor mRNA, a synthetic engineered pegRNA (epegRNA), and a nicking gRNA via electroporation. The insertion c.3569insG (exon 50) and the deletion c.4000delA (exon 52) were targeted with a PE3b and a PE3 system, respectively. Furthermore, both mutations were targeted simultaneously (combined approach). Prime-edited cells were analysed via immunofluorescence microscopy (IF), flow cytometric analysis, Western blot analysis (WB), ddPCR and next-generation sequencing (NGS).

Targeting exon 50 restored C17 expression in about 60% of treated primary JEB keratinocytes, shown by IF and flow cytometry. NGS analysis of these cells revealed that >95% of cells with restored C17 expression had a restored wild-type sequence, indicating a high amount of traceless gene correction. Restored protein expression of these cells comparable to wild-type level was further confirmed via WB. Here, C17 shedding was observed, indicating functional C17. NGS and ddPCR revealed wild-type RNA levels of >90% upon exon 50 targeting. Preliminary data for targeting of exon 52 showed a correction efficiency of around 30% in primary JEB keratinocytes, observed via IF and flow cytometry. Targeting both mutations in a combined approach, led to restored C17 expression in about 50% of cells, observed via IF and flow cytometry. In general, the combined approach showed higher expression levels of restored C17, presumably due to the correction of both alleles in some gene-edited cells.

In summary, PE proved to be efficient in the correction of two compound heterozygous *COL17A1* frameshift mutations associated with JEB, leading to restored C17 expression. This highlights the therapeutic potential of prime editing for patients with JEB.

P0568

Prime Editing advancements enable *in vivo* therapeutic correction of *ATP7B* p.H1069Q and p.R778L mutations in Wilson's disease

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Wilson's disease is a rare monogenic autosomal recessive disease caused by mutations in the *ATP7B* gene, which encodes a copper-transporting ATPase critical for copper homeostasis. These mutations manifest in disrupted copper metabolism resulting in copper accumulation in liver, brain and elsewhere. If left untreated, Wilson's disease can lead to serious complications such as liver failure, neurological damage, and decreased life expectancy. Current therapies are supportive and life-long with side-effects requiring intensive management. Therefore, there is a significant unmet medical need for a one-time, disease modifying therapy.

The *ATP7B* p.H1069Q and p.R778L mutations are the most common variants identified in the US/EU and East Asian populations, respectively, and represent more than 40% of Wilson's disease patients. Correction of the p.H1069Q and p.R778L mutations in Wilson's Disease patients by Prime Editing offers a potential curative and one-time therapeutic strategy.

We screened thousands of Prime Editors that correct the p.H1069Q and p.R778L mutations and identified potent, active, and highly precise editors with minimal unintended edits. In p.H1069Q patient-derived iPSC-differentiated hepatocytes, correction of p.H1069Q by Prime Editing restores physiological copper metabolism, similar to that observed in healthy human hepatocytes. To model Wilson's disease *in vivo*, we developed humanized *ATP7B* Wilson's disease mouse models that carry either the p.H1069Q or the p.R778L mutation, which recapitulate Wilson's disease phenotypes including copper accumulation and fibrosis in the liver, elevated urinary copper, and reduced serum ceruloplasmin activity. To deliver Prime Editing components *in vivo*, we developed a universal liver-targeted lipid nanoparticle (LNP) system formulated with lead Prime Editor components in the form of RNA (LNP-RNA PE) to correct p.H1069Q or p.R778L mutations. Following a single modest IV dose to humanized *ATP7B* mice, we observed more than 30% of alleles corrected in hepatocytes. These editing levels are expected to be disease-modifying. Using Prime Medicine's suite of biochemical and targeted sequencing assays, no off-target editing was detected in human hepatocytes for either the p.H1069Q or the p.R778L Prime Editors.

We have recently demonstrated the capability to Prime Edit the gene *SLC37A4* in the livers of adult cynomolgus monkeys using our universal liver-targeted LNP delivered by single IV infusion. It was well tolerated, with minimal transient cytokine or LFT changes and no histological changes. Use of a targeting ligand resulted in high levels of biodistribution to liver that correlated well with levels of Prime Editing. For Wilson's disease, we have developed a surrogate Prime Editor for nonhuman primate (NHP) studies that precisely edits at *ATP7B* p.H1069 that exhibits 40% precise editing in primary cynomolgus monkey hepatocytes. Additional NHP studies using this Prime Editor will be presented.

These results highlight that Prime Editing can precisely and efficiently correct the prevalent pathogenic mutations causing Wilson's Disease in the liver by LNP-RNA Prime Editors. Thus,

Prime Editing offers the potential of a safe and effective curative approach for Wilson's disease patients.

P0570

Highly efficient and seamless in vivo selection of long-range gene-edited HSPCs by targeting haploinsufficient genes

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Preclinical data in mouse models and occurrence of adverse events in some clinical trials have shown that random integration of strong promoters by viral vectors can be unpredictably genotoxic. We hypothesized that strong promoters may safely be exploited for clinical purposes when integrated into a single genomic locus, where genomic perturbations can be characterized beforehand.

While gene editing (GE) by homology-direct repair (HDR) allows for target integration of a therapeutic cassette into a validated locus of interest in hematopoietic stem and progenitor cells (HSPCs), it is presently constrained by low efficiency and risk of heterogeneous genetic outcomes. We thus developed an innovative strategy that intrinsically enriches HDR-edited HSPCs, while purging out genotoxic byproducts. This strategy is based on nuclease-based disruption of haploinsufficient loci in HSPCs, and reconstitution of the same loci by HDR. As the HDR cassette also includes the gene of interest (GOI), cells that survive the nuclease also harbor the GOI in the desired site.

Here we screened putative haploinsufficient loci and identified three candidate genes (*UBA1*, *RPS19* and *OGT*) whose knock-out significantly impairs the clonogenic output of edited HSPCs; upon transplantation into immunodeficient mice, the edited-HSPCs bearing indels are spontaneously counter-selected over time. In *UBA1* and in *RPS19* loci, we have obtained a proof of concept of selection of edited cells by HDR mediated integration of a "rescue" cassette also carrying a reporter GOI. As compared to the KO only counterparts, the HDR-edited HSPCs showed partial restoration of clonogenic output, and 80-90% of the colonies were reporter positive.

We then pursued in vivo validation for the target locus *UBA1*. While *UBA1*-KO HSPCs transplanted into NSGW mice resulted in very low engraftment in the bone marrow (<5% of live cells) as compared to a mock KO condition (55%), the HDR-edited cells in *UBA1* had an engraftment of nearly 40%, and nearly 100% of human cells were GFP positive. This can be compared to the commonly used AAVS1 safe-harbor target, where at comparable levels of HDR efficiencies (60% of cells) in vitro (input at transplant), only 40% of cells remained GFP+ at the experiment endpoint. Characterization of genomic and transcriptional perturbations at single-cell resolution driven by different strong promoters integrated in our candidate loci is ongoing.

We believe our strategy offers an appealing one-size-fits-all approach for HSPC gene therapy for metabolic diseases and beyond, allowing for targeted integration of a strongly expressed gene of interest into a well-known and characterized locus and spontaneous purge out of genotoxic editing byproducts.

P0571

One-pot PASTA: Precise and site-specific transgene addition by combining highly efficient CRISPR/Cas-mediated HDR and serine integrases in T cells

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Adoptive T cell therapy has emerged as a promising strategy for treating various malignancies. Despite significant advancements in genetically modifying T cells, current manufacturing methods, such as lentiviral transduction and CRISPR/Cas-mediated homology directed repair (HDR), are limited in their ability to efficiently and precisely integrate large DNA sequences into the genome, thereby restricting the therapeutic potential of engineered T cells. To overcome this challenge, we propose the utilization of site-specific integrases, particularly leveraging the inherent ability of large serine integrases, such as Bxb1, to facilitate recombination between sequence-defined attachment sites. Upon targeted insertion of an attachment site into the genome, DNA vectors containing the corresponding attachment site can be introduced through integrase-mediated recombination at the prespecified site. Previous studies have introduced techniques such as PASSIGE and PASTE, which combine prime editing with site-specific integrases to “paste” larger genetic cargo into cells. However, achieving efficient insertion of landing pads via prime editing in primary human T cells remains challenging, hindering the adoption of these methods and ultimately limiting the efficacy of integrase-mediated recombination.

We have developed “one-pot PASTA” (precise and site-specific transgene addition), a versatile CRISPR-integrase system combining highly efficient CRISPR/Cas-mediated HDR for precise landing pad insertion with site-specific recombination via serine integrases in a single transfection. This approach enables incorporation of large, multicistronic DNA constructs in a site-specific manner. Achieving maximum editing efficiencies depended significantly on refining the delivery of our CRISPR-integrase system, involving fine-tuning the ratios of its components for optimal performance. Comparative studies with standard CRISPR/Cas-mediated knock-in methods, reliant solely on HDR, demonstrate that our CRISPR-integrase system significantly outperforms traditional methods in efficiently inserting large transgenes exceeding 6 kb insert size.

Using “one-pot PASTA”, we targeted the T cell receptor alpha constant chain (*TRAC*) gene locus in primary human T cells for precise delivery of multicistronic constructs. Integration of a CD19-directed second-generation chimeric antigen receptor (CAR) enabled expression under the control of the endogenous *TRAC* promoter, resulting in physiological CAR regulation which was shown to improve functionality and reduce exhaustion. Additionally, we co-integrated an exogenous promoter downstream of the CAR for constitutive overexpression of transgenes aimed at augmenting T cell function and safety. To this end, we designed constructs to co-express safety switches and cytokine receptors. Unexpectedly, we observed inadequate transgene expression

via the exogenous promoter integrated at the *TRAC* locus, requiring systematic optimization of construct designs to achieve sustained transgene expression.

The CRISPR-integrase system ("one-pot PASTA") represents a flexible platform for precise and efficient insertion of gene-sized cargo in T cells, providing a simple and straightforward alternative to emerging prime editing based methods, such as PASSIGE and PASTE. By leveraging serine integrases to incorporate larger genetic payloads into primary human T cells, we can engineer T cells with designed capabilities and synthetic circuits, increasing their therapeutic potential and safety within clinical applications. In the future, "one-pot PASTA" may also be used in T cells and different cell types to address other diseases with unmet clinical needs, such as genetic disorders caused by large deletions.

P0572

Senescence and inflammation are unintended adverse consequences of CRISPR-Cas9 gene editing in human hematopoietic stem cells

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Gene editing (GE) via homology-directed repair (HDR) in hematopoietic stem and progenitor cells (HSPCs) represents a promising strategy for site-specific gene correction of several inherited diseases. However, its clinical translation is still hampered by limited efficiency and the emergence of adverse cellular responses constraining HDR-edited HSPC repopulating potential upon transplantation. We previously showed that the combination of nuclease-induced Double Strand Break (DSB) with DNA repair template for HDR delivered by AAV6, caused cumulative activation of the p53-mediated DNA Damage Response (DDR) pathway, constraining HSPC proliferation and yield. This suggests that DDR-related cellular programs may inadvertently contribute to HSPC dysfunction upon GE. Protracted DDR signalling has been linked to the establishment of cellular senescence, a condition in which cells, despite being still alive, are unable to further proliferate and are characterized by activation of inflammatory programs. Yet, whether GE could have durable and long-term consequences on the functionality of HSPCs remains to be elucidated. The overreaching goals of the project are to uncover the long-term impact of CRISPR-Cas9 GE in cord blood (CB)- and mobilized peripheral blood (mPB)-derived HSPCs and to develop new strategies to counteract edited HSPC dysfunction for more efficient gene and cell therapies. By integrating transcriptional analysis (up to the single cell level) with innovative imaging-based cellular assays, we reported induction of cellular senescence markers (p16 and Senescence-Associated β-Galactosidase) and pro-inflammatory programs across edited HSPC subtypes and *in vivo* in the human graft. Interestingly, we found transcriptional upregulation of several senescence-associated and inflammatory genes of the *IL1 axis* (an upstream mediator of DDR-dependent inflammation) and NF-kB pathway (a key regulator of inflammatory genes) especially in HDR-edited cells. Transcriptional activation of inflammatory cytokines in edited HSPCs was DDR-dependent and partly mitigated by transient p53 inhibition, with consequent improved polyclonal reconstitution of genetically engineered HSPCs in the long-term, albeit at the cost of a higher mutational burden. Instead, senescence modulation by anti-inflammatory

treatments (specifically by temporary inhibition of *IL1* and NF-kB pathways at the time of GE) resulted in increased clonogenicity of edited HSPCs *ex vivo* and rescued robust clonal output in repopulating HSPCs *in vivo*, without aggravating the genotoxicity risks of the GE procedure. Our findings define senescence and inflammatory programs as long-term uncharted barriers to efficient GE and pave the way for more efficient and safer strategies, based on senescence modulation and anti-inflammatory molecules, for HSPC-based clinical application.

P0573

Targeted T cell expressed Diacylglycerol Kinase (DGK)s gene editing enhances Chimeric Antigen Receptor (CAR) T cell durability and efficacy against cancer

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Chimeric Antigen Receptor (CAR) T cells, engineered to target specific antigens and trigger immune responses, offer a revolutionary approach to cancer treatment. We previously reported that targeted gene editing of T cell expressed Diacylglycerol Kinase alpha and zeta (DGKA and DGKZ) enhances CAR-T activity against solid tumor by offering resistance against TGFB and PGE2. One of the other main challenges that CAR-T faces is T cell dysfunction due to chronic stimulation of CAR. We here asked ourselves whether targeted gene ablation of DGKA and DGKZ can hamper T cell dysfunction under repeated CAR antigen challenges. For this, we utilized well characterized anti-CD19 41BBz and 28z 2nd generation CAR with or without DGKA/DGKZ gene editing and performed in vitro and in vivo repeated CD19+ tumor challenges. We found DGKA/DGKZ gene edited anti-CD19 CAR-T retained T cell growth, pro-inflammatory cytokine release and tumor killing activity upon in vitro repeated tumor antigen challenge in DGKA/DGKZ edited groups in both 41BBz and 28z CAR when compared to control group. Furthermore, this finding corroborated with in vivo where DGKA/DGKZ gene edited anti-CD19 CAR maintained tumor killing activity even after repeated tumor challenge. To further confirm these findings in molecular level, we performed RNA-seq from samples that were undergone repeated tumor antigen challenge in vitro and found DGKA/DGKZ gene edited CAR-T showed transcriptomic changes that are associated with cell proliferation and T cell memory phenotypes along with pro-inflammatory cytokines and chemokines. These findings suggest that targeted gene editing of DGKA and DGKZ in engineered T cells can not only offer resistance to tumor microenvironment but also improve durability.

P0574

Monitoring repair outcomes of CRISPR genome-editing at the single-cell level

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CRISPR-Cas genome-editing technology is rapidly integrated in diverse clinical applications for the treatment of various diseases. Editing of genomic targets is achieved by using specifically designed guide RNAs (gRNAs) coupled with a nuclease, which cuts at the target sequence and induces targeted editing. However, in addition to the desired on-target editing, the system might also cause unintended effects in both on- and off-target sites, including insertions and deletions (inDels) and structural variations (SVs) such as translocations and inversions. Furthermore, even if exclusive on-target editing is achieved, not all edits contribute to the desired phenotype. Current gold-standard approaches for on- and off-target activity measurement involve a two-step methodology: first identifying potential off-targets, and then quantifying editing frequency at the nominated sites. However, these bulk-sequencing-based assays lack sufficient sensitivity to characterize editing outcomes at the resolution of individual cells, thus cannot provide information on the proportion of engineered cells with the desired phenotype. To address this gap, we have harnessed the Tapestri technology, a single-cell DNA sequencing (scDNA-Seq) platform, which enables the sequencing of CRISPR-edited cell products at a single-cell level. This technology offers high-resolution insights into the co-occurrence of on- and off-target editing, zygosity of editing profile, and cell clonality assessment. Furthermore, unlike conventional amplification-based bulk sequencing methods, which only report SVs presence, single-cell data enables the capture of the SVs frequency while integrating inDels data. This enhanced resolution is especially crucial for genome-editing designs with multiple targets, to ensure accurate editing at all specified sites. Hence, we have engineered human primary T-cells using a multiplex three gRNAs complex, designed to knock-out the *PDCD1*, *TRAC*, and *TRBC* anti-cancer targets. To address off-target activity, we created a 111-site panel based on prior GUIDE-seq experiments, and sequenced these sites using the Tapestri platform, generating output data of ~10,000 edited cells per sample. Next, we evaluated the inDel profile at each allele. Interestingly, different gRNAs showed different ratios of mono-allelic versus bi-allelic editing. Furthermore, only a minority of the cell population harbored bi-allelic frameshift mutations across all on-target sites without exhibiting off-target genotoxicity. Single-cell analysis also revealed a heterogenous spectrum of CRISPR outcomes in edited cells, highlighting the importance of thoroughly characterizing the genome-engineered cell product. We also identified translocations between on-target and off-target loci, including a novel translocation that was not detected in previous studies. CRISPR editing-activity and translocations results were validated using bulk DNA sequencing using rhAmp-Seq technology, attesting to a high degree of reproducibility. In summary, single-cell DNA sequencing for assessing genome-editing outcomes surpasses existing standards by (1) facilitating precise measurement of editing activity per-cell and per-allele at on- and off-target sites; (2) providing comprehensive co-occurrence profiling of editing outcomes in the individual cell level; and (3) enabling sensitive SVs quantification. This high-resolution methodology represents a leap forward in our understanding of CRISPR outcomes, while also serving as a platform for a highly sensitive quality control evaluation of genome-engineering products, holding the potential to enhance and optimize therapeutic applications of the CRISPR-Cas platform.

P0575

Development of PM359, a Prime Edited CD34+ cell drug product for the treatment of p47phox Chronic Granulomatous Disease

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P47 phagocytic oxidase Chronic Granulomatous Disease (p47phox CGD, hereafter CGD) is an inherited immunodeficiency caused by mutations in *NCF1* which encodes p47 protein, a subunit of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. When neutrophils encounter pathogens, NADPH oxidase produces superoxide that kills pathogens. CGD patients lack NADPH oxidase, which leads to recurrent infections and inflammation. Allogeneic hematopoietic stem cell transplantation (HSCT) is the only available potential cure, but patients may not be eligible. CGD is caused by a delGT mutation in *NCF1*. In the context of developing a Prime Edited (PE) CD34+ cell therapy for CGD (PM359 hereafter), nonclinical pharmacology studies were conducted in which a Prime Editor (PE) was designed to precisely correct the delGT mutation at *NCF1* in CGD CD34+ cells to restore neutrophil NADPH oxidase activity.

PE components manufactured under Good Laboratory Practice (GLP) process were tested in CD34+ cells from three CGD patients. Greater than 80% CD34+ cells had at least 1 delGT allele corrected (84% ± 9) with no significant difference in viability between PE and Mock treated cells (PE: 86% ± 6; Mock: 85% ± 5). An *in vivo* study was conducted with PE and Mock CGD CD34+ cells to compare long-term HSC (LT-HSC) engraftment, on-target Prime Editing, off-target editing, transcriptional profiles, and restoration of neutrophil NADPH oxidase activity. At week 16 post HSCT in immunodeficient mice, 80% of bone marrow PE CGD CD34+ cells had at least 1 delGT allele corrected, 81% of PE CGD neutrophils had restored NADPH oxidase activity with no significant differences between PE and Mock treated CGD CD34+ cells with respect to hematopoietic reconstitution. Clinical pathology and genotoxicity assessments indicated no adverse clinical observations, no tumors, no off-target editing events, and no chromosomal rearrangements. Transcriptome analysis showed that PE CGD cells had reduced expression of interferon pathway genes compared to Mock CGD cells, suggesting resolution of inflammation by correction of the CGD causative mutation.

Toward development of PM359, a clinical scale GMP manufacturing cell process was developed with Healthy Donor CD34+ cells (HD drug product, HDDP) and PM359 GLP PE components. Four exemplary HDDP batches were transplanted into immunodeficient mice, with no significant differences observed between PE and Mock CD34+ cells 16 weeks post HSCT with respect to hematopoietic reconstitution. A 2˚ HSCT study conducted to evaluate durability of PE LT-HSC showed no significant differences between PE and Mock treated cells with respect to hematopoietic reconstitution. Greater than >70% of LT-HSCs had at least 1 Prime Edited allele after 1˚ and 2˚ HSCT (24 weeks post initial cell infusion into 1˚ recipients). Clinical scale lot comparison of CD34+ cells from four HDDP lots yielded similar CD34+ cell purity (98±1%), PE efficiency (74±8% of CFCs with at least 1 allele corrected), and post-editing cell viability (78±3%). These findings show that correction of the delGT mutation in CGD patient CD34+ cells may provide a safe and efficacious curative treatment for CGD patients. An Investigational New Drug application for PM359 for the treatment of CGD was cleared by the FDA in April 2024.

P0576

Efficient correction of the C282Y mutation in a murine model of hereditary hemochromatosis by lipid nanoparticle/mRNA-mediated base editing

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Hereditary hemochromatosis is one of the most prevalent metabolic diseases in the European population, affecting 1:200 to 1:500 individuals. More than 80 % of the cases are caused by a homozygous single missense substitution (p.Cys282Tyr) in the homeostatic iron regulator (*HFE*) gene, which leads to excessive iron absorption and accumulation of iron in the organs and tissues. Affected patients may develop liver cirrhosis, diabetes mellitus and cardiomyopathy. The treatment of hemochromatosis involves lifelong phlebotomy and the use of chelating agents. Currently, the only cure of the disease is liver transplantation, but gene therapy strategies hold great promise for the future.

Adenine base editing, one of the latest CRISPR/Cas9-derived technologies, can be used to precisely convert A to G in genomic DNA without inducing DNA double-strand breaks. We have previously identified an efficient single-guide RNA (sgRNA) targeting the most common hemochromatosis mutation C282Y using adenine base editing and demonstrated successful genomic correction in the 129-Hfe tm.1.1Nca hemochromatosis mouse model (Rovai *et al*., Nature Communications 2022). Using an AAV8 split vector system for delivery, we were able to achieve an A>G correction of 12 % in the whole liver and an improved iron metabolism four months after treatment. In our current study, we have progressed from viral vectors to mRNA/lipid nanoparticle (mRNA/LNP) technology to improve efficiency, avoid long-term adenine base editor expression and move one step further towards clinical application. In cooperation with Acuitas Therapeutics, we encapsulated ABEmax mRNA and the highly modified sgRNA into LNPs for liver-specific delivery. *In vitro* transfection of primary mouse hepatocytes from 129-Hfe tm.1.1Nca mice resulted in 74 % A>G correction. *In vivo*, we achieved A>G correction of 34 % in the whole liver and 50 % in isolated hepatocytes one month after administration of 2 mg/kg mRNA/LNP. Analysis of the inflammatory liver enzymes AST and ALT in serum samples collected 24 hours after administration revealed a moderate increase in female animals, while male mice showed no significant increase compared to a PBS control group.

In conclusion, we demonstrate that our base editing approach has the potential to be a safe and efficient option for a future curative therapy of hereditary hemochromatosis.

P0577

A precise and efficient genome editing strategy for *ELANE*-associated severe congenital neutropenia using an adenine base editor

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Severe congenital neutropenia (CN) is an inherited pre-leukemia bone marrow failure syndrome commonly caused by autosomal-dominant *ELANE* mutations (*ELANE*-CN), encoding neutrophil elastase (NE) protein. CN patients suffer from severe, life-threatening bacterial infections starting early after birth due to the absence or very low numbers of neutrophils in the peripheral blood. Daily injection of recombinant human granulocyte colony-stimulating factor (rhG-CSF) is the standard treatment option for CN patients. However, not all patients respond to rhG-CSF and approximately 15 % of *ELANE*-CN patients develop myelodysplastic syndrome (MDS) or acute myeloid leukemia (AML). Hematopoietic stem cell transplantation (HSCT) is an ultimate curative option for CN patients, but it carries risks of adverse events and possible mortality. Disease phenotype correction by gene editing of patients` $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) *ex vivo*, followed by autologous transplantation, may offer an alternative curative therapy. Here, we report the development of a gene editing approach for *ELANE*-CN through inhibition of the *ELANE* mRNA expression by converting two adenine nucleotides to guanine nucleotides in the *ELANE* promoter TATA box (TATA > TGTG) using CRISPR/Cas9 adenine base editor (ABE). We found that modifying TATA box using mRNA ABE in the THP-1 reporter cell line led to markedly reduced *ELANE* expression levels up to 70% five days post-electroporation, as assessed by measuring the luminescence levels of endogenously tagged NE protein and qRT-PCR for *ELANE* mRNA expression. The on-target editing efficiency was 97%, as assessed by analysis of Sanger sequences traces five days post-electroporation. ABE editing of *ELANE*'s TATA box did not disrupt granulopoiesis in healthy donor HSPCs, while there was a markedly elevated neutrophil differentiation in gene-edited *ELANE*-CN patient's HSPCs (n = 2), as assessed by CFU assay or liquid culture granulocytic differentiation and compared to the mock electroporated group. Furthermore, the *in vitro* generated neutrophils were functional, being able to produce reactive oxygen species (ROS) upon activation with N-Formyl-Met-Leu-Phe (fMLP) and perform phagocytosis of pHrodo green Staphylococcus aureus bioparticles. We are currently employing CAST-seq, rhAMP-seq and optical genome mapping, to assess the safety profile of *ELANE*'s TATA box base editing of HSPCs. In conclusion, our findings demonstrate the potential of *ex vivo* CRISPR-Cas9 adenine base gene editing as a curative therapy for *ELANE*-CN patients. This innovative approach offers a double-strand break-free strategy that precisely targets the regulatory TATA box of the *ELANE* gene promoter without modifying coding sequence and restoring granulopoiesis by reducing the expression level of the disease-causing gene. This approach has the potential to find a wide range of clinical applications in the future.

P0578

Exploring the roles of DNA repair processes across diverse Prime Editing strategies with pooled screens and Knock-Knock Prime

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Prime Editing allows for the precise installation of a wide range of programmed changes to genomic sequence. DNA mismatch repair (MMR) has previously been identified as an important cellular pathway that influences Prime Editing outcomes in some cell types. In standard models, heteroduplexes formed as intermediates during Prime Editing can be recognized and reverted by MMR, potentially requiring multiple rounds of flap reverse transcription before an edit is successfully installed. To alleviate this bottleneck, inhibition of MMR activity or evasion of MMR by co-installation of additional edits have been explored, but the activity of MMR on different types of heteroduplexes in human cells remains incompletely understood. To systematically explore the role of MMR and other DNA repair processes during Prime Editing, we performed pooled screens of large sets of pegRNAs programming a diverse range of substitution, insertion, deletion, and combination edits in MMR-competent cells. To leverage the rich datasets produced by these screens, we developed an improved computational pipeline called Knock-Knock Prime for quantifying the full spectrum of Prime Editing sequence outcomes. We identified substantial diversity between the average efficiency of different substitution types and between insertions and deletions. To understand the DNA repair mechanisms that underlie these differences, we performed genetic screens in which diverse types of Prime Editing were carried out in the presence of individual DNA repair gene knockouts in a pooled library format. Applying Knock-Knock Prime to screening data highlighted striking differences between the genetic dependencies of different edit types, producing a high-resolution map of the division of labor between different DNA repair sub-pathways during Prime Editing. Our results both inform the design of improved Prime Editing strategies and highlight how the ability of Prime Editing to create arbitrary heteroduplexes represents a powerful tool for understanding the basic biology of DNA repair processes. Moreover, our development of Knock-Knock Prime offers a robust and general approach for characterizing Prime Editing outcomes and is particularly useful for analyzing outcomes for complex edit types.

P0579

Tridimensional scaffolds enable efficient and safe genetic engineering of hematopoietic stem and progenitor cells across multiple gene therapy platforms

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Hematopoietic Stem and Progenitor Cells (HSPC) engineering, and particularly long-range gene correction, requires *ex vivo* activation to achieve a sizable fraction of edited cells. However, we recently discovered that *ex vivo* culture inadvertently leads to p38/ROS-dependent proliferative stress and DNA damage, resulting in stem cell dysfunctions and potentially jeopardizing the efficacy and safety of genetic engineering for HSPC-based clinical applications. We reasoned that these detrimental responses may arise from HSPC removal from the bone marrow niche and be exacerbated by growth on bidimensional substrates. Thus, we employed a biocompatible tridimensional scaffold to counteract culture stress and reported mitigation of DNA damage accumulation, increased clonogenic potential, and enhanced multi-lineage repopulating capacity of HSPC upon transplant into immunocompromised mice. Transcriptomic analyses revealed massive transcriptional changes upon 3D culture, including upregulation of genes belonging to cytoskeleton organization and adhesion and concomitant downregulation of oxidative phosphorylation and protein synthesis, features associated with more primitive stem cells. Mechanistically, 3D scaffolds prevented HSPC deformation and squashing occurring in bidimensional culture, conserving nuclei morphology and tubulin polarity, potentially facilitating adaptation to *ex vivo* culture and preservation of stemness properties. Most importantly, pre-culture on 3D scaffolds ameliorated HSPC functionality upon Cas9/AAV6-mediated GE, endowing edited cells with increased multi-lineage output at single-cell resolution *in vitro* and superior long-term and polyclonal engraftment upon secondary transplant into immunocompromised mice. Similar results were obtained across different HSPC sources, such as cord blood and mobilized peripheral blood, as well as in multiple therapeutic settings, including lentiviral gene transfer and HSPC expansion, which is particularly relevant for certain diseases in which only a limited number of cells can be harvest from patients. Finally, we implemented our 3D scaffolds into the clinically approved gene therapy protocol for Wiskott-Aldrich Syndrome using patient-derived samples and GMP-compliant vectors and reagents and reported increased clonal output for 3D cultured-engineered cells. Overall, our results highlight the importance of tridimensional interactions for the maintenance of HSPC biological properties during *ex vivo* manipulation for therapeutic applications. We propose a ready-to-go strategy that can be directly implemented into clinically approved gene therapy protocols, minimizing detrimental cellular responses during *ex vivo* engineering and increasing the efficacy and safety of these treatments.

P0580

Stepwise optimization of the gene editing protocol for the clinical translation of RAG1 correction in human hematopoietic stem and progenitor cells

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Recombination activating gene 1 (*RAG1*) orchestrates V(D)J recombination and it is essential for lymphopoiesis and somatic diversity of the T cell receptor and B cell receptor. Mutations cause a broad spectrum of severe immunological disorders including T-B- Severe Combined immunodeficiency (SCID) due to null mutations, Omenn Syndrome and other leaky forms caused by hypomorphic *RAG1* mutations. Curative treatment is hematopoietic stem cell transplantation but limited by donor availability and conditioning-related toxicity. Because of the tight regulation of *RAG1* gene expression during lymphoid differentiation, we recently developed an alternative therapeutic option based on the *in situ* correction aimed at preserving the endogenous control. We demonstrated the efficacy of our combined "knock-out / knock-in" gene editing strategy to rescue the physiological *RAG1* expression and function. Importantly, homology directed repair (HDR)-mediated gene editing enabled the correction of human *RAG1* in hematopoietic stem and progenitor cells (HSPC) obtained from patients with hypomorphic RAG1 mutations and allowed the overcome of T and B cell differentiation blocks. These findings support the clinical translation of HSPC gene editing for the treatment of RAG1 deficiency. To move towards the clinical-grade process development, we optimized our research-grade gene editing protocol (standard protocol) in terms of culture conditions, delivery platforms and safety. Having previously defined the best performing set of single guide RNA (sgRNA) and corrective donor templates to successfully edit the *RAG1* locus, we tested our gene editing platform exploiting GMP cell culture medium and cytokines combined with a clinical-grade electroporator system. Interestingly, in small-scale *in vitro* experiments, the clinical-grade protocol, exploiting the selected gRNA and adeno-associated virus 6 (AAV6) donor, enabled a two-fold increase of HDR efficiency in human mobilized HSPC as compared to the research-grade protocol, further exceeding the minimal therapeutic threshold of correction. Cell fitness analyses showed an improved cell growth rate of HSPC edited with the clinical-grade protocol associated with a higher proportion of most primitive HSPC (CD34+CD133+CD90+) as compared to the standard protocol. Moreover, to mitigate the adverse cellular impact and potential genotoxic risk associated to AAV6-mediated template delivery in HSPC, we combined the clinical-grade protocol with the Integrase Defective Lentiviral Vector (IDLV) as donor template and assessed cell fitness and HDR efficiency. Preliminary data confirmed the improved HSPC growth and phenotypic profile obtained with the clinical-grade protocol and IDLV delivery, resulting in an increased yield of edited HSPC *in vitro*.

P0581

An all-Prime Editing one-step approach for non-viral generation of a multiplex-edited allogeneic CAR-T cell drug product

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Despite the success of CAR-T cell therapy for treatment of lymphoma and multiple myeloma, several challenges limit broad clinical application of this therapy, including manufacturing cost, timelines, and variability in drug product quality and potency due to the autologous nature of commercially available CAR-T drug products (DP). DP potency and safety profiles are also variable due in part to viral vectors and transposons used for engineering T cells, for which the integration site and transgene copy number cannot be controlled. In contrast, Prime Editing (PE) can be used for precise, site-specific programmable gene editing. Prime Editing is also able to precisely

integrate large genetic cargo into specific genetic loci with Prime-Assisted Site-Specific Integrase Gene Editing (PASSIGE). By combining PE-mediated gene knockout and PASSIGE, PE can create multiple edits in a single step, potentially allowing production of a safe, effective, potent, and cost-effective allogeneic 'off the shelf' (allo-OTS) CAR-T DP. Clinical scale production of a multiplex Prime Edited (PE) allo-OTS from healthy donor T cells would potentially increase quality and reproducibility and eliminate patient-relevant DP variability which has been observed in some autologous CAR-T DP.

Toward production of a multiplex PE allo-OTS CAR-T cell therapy, a strategy was designed to knockout multiple genes involved in alloreactivity and T cell regulation by PE in combination with PASSIGE integration of a CD19 specific CAR (CD19-CAR) at the *TRAC* locus. Prime Editors were developed to knock out expression of the T cell receptor (*TRAC*), MHC Class I and II (*B2M* and *CIITA*), and the immune checkpoint PD1 (*PDCD1*). Comprehensive high throughput screening identified efficient Prime Editors that precisely disrupted expression of targeted genes in a single step in >90% of human T cells after delivery of PE components to the cells. Importantly, there were no significant differences between PE and mock treated control T cells with respect to viability, expansion, and basic T cell functional properties. Co-delivery of these Prime Editors, Bxb1 recombinase, and a nonviral DNA donor template resulted in targeted PASSIGE integration of a 3.5 kb CD19-targeting CAR at the *TRAC* locus in over 80% of the T cells, with no observed impact on T cell viability. PASSIGE-generated CD19 CAR-T cells show potent anti-tumor activity and cytokine production in response to $CD19^+$ tumor cell lines *in vitro* and *in vivo*. The PE and PASSIGE platform was further optimized to achieve efficient multiplex editing across five target sites in one delivery step (>70% of T cells contain multiplex edits) and integration of large genetic cargo (>50% of T cells expressed all transgenes in the 10 kb expression cassette after site-specific integration). These findings demonstrate the flexibility, versatility, and precision of the PE and PASSIGE platform in producing multiplex edited engineered T cells without the need for viral vectors.These developments in the PE and PASSIGE modular programmable platform can potentially enable production of the next generation precision allo-OTS T cell therapies for broad application in the treatment of cancer, inherited and acquired diseases.

P0582

In-vitro and *in-vivo* studies to support SIGHT-I clinical trial, the world's first CRISPR/RNA-targeting therapy of HG202, for patients with neovascular age-related macular degeneration

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Age-related macular degeneration (AMD) is a progressive disease that causes severe central vision impairment in adults over the age of 50. Neovascular AMD (nAMD) accounts for 90% of AMD-related cases of blindness. The current gold-standard treatment for nAMD is frequent, invasive intravitreal injections of anti-vascular endothelial growth factor (VEGF) agents by blocking VEGF proteins, which leads to severe complications and reduces patient adherence to

treatment, resulting in vision loss over time. Additionally, up to 40% of patients fail to respond or only partially respond to anti-VEGF therapies. Here, we developed HG202 using high-fidelity CRISPR/Cas13 RNA-targeting technology packaging into a single adeno-associated virus (AAV) vector to knock down the VEGFA mRNA expression in the retina partially and conducted an open-label, multicenter, and dose-escalation **SIGHT-I** study (NCT06031727) to investigate the safety, tolerability, and efficacy of a single subretinal injection of HG202 in patients with nAMD. Over 40% of *Vegfa* mRNA expression in the retina of mice (n=8) was suppressed after injecting HG202. The area of laser-induced choroidal neovascularization (CNV) (87% reduction, p <0.0001, n=14) was reduced in HG202-treated mice. Comparatively, the inhibition efficiency of AAV-anti VEGF was 70% reduction (p <0.01, n=14) when compared to the vehicle control; Aflibercept showed 47% reduction (p <0.01, n=18) when compared to HG202. A single injection of HG202 achieved a durable therapeutic effect for at least 13 weeks in the laser-induced CNV mouse model. No systemic toxicity or immunotoxicity was observed in NHPs. Based on the preclinical findings, nine nAMD patients who are either non-responsive and resistant to anti-VEGF therapies or responsive to anti-VEGF therapies but require frequent injections with Early Treatment Diabetic Retinopathy Study (ETDRS) best-corrected visual acuity (BCVA) between 73 and 23 in the study eye at Screening will be randomized to one of the three doses of HG202. The primary objectives are the safety and tolerability of HG202 at different doses. Secondary objectives include mean change in BCVA, central retinal thickness (CRT), and other efficacy endpoints. As of May 2024, 4 subjects have been enrolled in the low- and middle-dose cohorts. Subject #202 (the world's first individual receiving novel CRISPR/Cas13 RNA-targeting therapy), a non-responsive and resistant to anti-VEGF therapies patient, had a history of 6 anti-VEGF injections within 8 months with persistent macular fluid before the injection. Subject #202 baseline included CRT of 233 μm and BCVA of 29 letters. Retinal fluid was significantly eliminated post-injection, and CRT was recovered to 162 and 155 μm at 4- and 8-week post-injection, respectively. The BCVA improvement of nearly 15 letters was observed starting at 4 weeks and sustained at 16 weeks post-injection visit. No drug-related serious adverse event has been observed in all four treated subjects. Four subjects with nAMD had an acceptable local and systemic adverse-event profile after the delivery of HG202 in the **SIGHT-I** study. Therefore, HG202, as a novel therapy, has the potential to provide safe and effective RNA-targeting therapy to treat nAMD patients who are non-responsive or develop tachyphylaxis to anti-VEGF agents with a one-time treatment.

P0583

A Transformative DMD cytosine base editing drug

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Duchenne muscular dystrophy (DMD) is the most severe form of muscular dystrophy affecting 1 in 3500 to 5000 boys at birth globally. Patients experience a progressive wasting of both skeletal and cardiac muscles attributable to the loss of dystrophin protein encoded by the DMD gene on chromosome Xp21. The milder symptoms in Becker muscular dystrophin (BMD) patients, who has an in-framed truncated but still functional dystrophin protein, provides a hereditary basis for exon skipping therapy against DMD. GEN6050 is a preclinical investigational drug which utilized Targeted AID-mediated Mutagenesis (TAM) cytosine base editor (CBE) to induce DMD exon 50 skipping via editing the 5'SS of DMD IVS50. GEN6050 is an intravenous dual AAV product containing two AAV9 drug products. One encodes muscle-specific promoter-driven TAM CBE protein while the other encodes 3 copies of hE50 sgRNA targeting the junction between DMD exon 50 and intron 50. Also, the sgRNA vector carries a human gamma-actin gene, which can bind de novo Dystrophin to rapidly facilitate costamere and Dystroglycan-associated complex remodeling and provide a synergistic therapeutic effect with TAM CBE. In vitro, GEN6050

exhibited high editing efficiency on target sites in different DMD iPSC-derived cardiomyocytes and myotubes. In vivo, the mouse surrogate demonstrated therapeutic effects of TAM CBE in a DMD E4* mice model with significant CK-MB reduction and the substantial pathway recovery in heart accompanied by the restoration of Dystrophin protein. The MOA of GEN6050 was confirmed in wild-type mice, which caused dose-dependent and time-accumulated editing efficiency and exon skipping. The proof-of-concept study using humanized GEN6050 is undergoing. The offtarget assessments in normal iPSC-derived cardiomyocytes or myotubes showed low to undetectable sgRNA-dependent or independent offtargets. No transcriptome offtargets were found, confirming that TAM CBE is an RNA edit-free DNA editor. We have successfully established the large-scale AAV9 process development using triple transfection, suspended HEK293 (vpc2.0) system. The IND will be filed at the end of 2024. Furthermore, an investigator- initiated trial will begin in China in June. In sum, GEN6050X has demonstrated therapeutic potential in vitro and in vivo and may provide a cure to DMD exon 50 skipping patients.

P0584

Efficient and sustainable multi-gene silencing by epigenetic modulation enhances the functionality of universal CAR-T cells

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Gene editing approaches have been widely used in T cell-based therapies, such as CAR-T, TCR-T, and TILs, to enhance anti-tumor performance *in vivo*. However, potential genetic instability concerns are raised using DNA cleavage-mediated gene editing, particularly when multiplexed gene editing is introduced to allogeneic CAR-T cell therapy. Here, we employed EPIREG®, an epigenetic modulation technology composed of a nuclease-independent DNA binding domain fused with DNA methylation and histone modification effectors, for efficient, durable, and specific silencing of allogeneic target genes as well as immune checkpoint genes in T cells.

The EPIREG® mRNA and guide RNA (sgRNA) targeting *TGFBR2* were delivered into human primary T cells using electroporation. We found that transient expression of EPIREG® suppressed *TGFBR2* mRNA and protein expression levels by over 90%, sustained for more than four weeks. Additionally, we examined the possibility of EPIREG® in simultaneously silencing multiple genes in primary T cells, and found that EPIREG® achieved robust silencing efficiency (>90%) on up to seven genes simultaneously, similar to that achieved by targeting individual genes separately. Furthermore, whole-genome bisulfite sequencing (WGBS) and CUT&Tag analyses at 7- and 21- days post-electroporation demonstrated that EPIREG® achieved durable transcriptional suppression by introducing DNA methylation and reducing activation-related histone modifications at target sites.

Whole-genome and transcriptome wide analyses revealed that EPIREG® treatment induced no off-target DNA methylation, histone modifications, or gene expression alterations compared to the control group. Karyotype analysis demonstrated that EPIREG® showed no influence on chromosome integrity given the cleavage-free nature of epigenetic editing. By contrast, CRISPR/Cas9 editing caused significant chromosomal aberrations (10% in single gene and 27% in

seven gene-edited groups). Further PEM-seq analysis demonstrated that CRISPR/Cas9 editing targeting *TGFBR2* induced chromosomal translocations in over 8% of primary T cells, and many of these translocations occurred within oncogenes and tumor suppressor genes. In addition, multiple gene-edited T cells by CRISPR/Cas9 exhibited reduced cell proliferation and viability, which was not observed in those treated by EPIREG®. These results highlighted the minimal off-target effects and superior safety of EPIREG® in engineering T cells.

We next examined the potential of EPIREG® improving the functionality of clinically used UCAR-T cells. We first generated dual-target modified CAR-T cells by silencing both *TGFBR2* and *CD38* genes in CD19-CAR-T cells and found that the dual-target modified CAR-T cells exhibited enhanced anti-tumor activity both *in vitro* and *in vivo*, with improved cytokine secretion, increased stemness, and greater cytotoxicity against tumor cells. Furthermore, EPIREG® achieved over 99% suppression on TCR expression and T-cell-resistance-related targets including MHC I and MHC II in UCAR-T cells for over five weeks, facilitating the creation of potent allogeneic CAR-T cells without safety concerns. By simultaneously silencing TCR, MHC I, MHC II, and *TGFBR2*, EPIREG® enabled the production of highly functional UCAR-T cells, significantly enhancing their therapeutic potential while minimizing the risks of graft-versus-host disease and immune rejection.

In summary, EPIREG® achieved efficient, sustainable, and specific gene suppression in T cell therapies, providing a safer and more efficient alternative in developing UCAR-T cells for future innovations in cell-based immunotherapies.

P0585

Circular Single-Stranded DNA Enables Efficient TALEN-Mediated Gene Insertion in Long Term HSC

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Gene therapy strategies utilizing hematopoietic stem and progenitor cells (HSPCs) hold the promise of delivering a lifelong supply of therapeutic agents. Gene editing enhances these therapeutic prospects by employing engineered nucleases to introduce sequence-specific double-strand breaks (DSBs) at targeted genomic loci. This facilitates the knockout (KO) of genes or the knock-in (KI) of therapeutic genes in the presence of a DNA donor template. While viral vectors, particularly Adeno-associated viruses (AAVs), are prevalent carriers of donor templates, non-viral alternatives such as linear single-stranded DNA (LssDNA) and circular single-stranded DNA (CssDNA) are emerging as promising options.

Capitalizing on TALEN® technology, we have devised a gene editing process that incorporates non-viral DNA donor template delivery (LssDNA or CssDNA) to enhance gene insertion in hematopoietic stem and progenitor cells (HSPCs). Our findings indicate that CssDNA yields a 3- to 5-fold higher gene insertion frequency than LssDNA, with efficiencies surpassing 40%. Notably, this correlates with increased cell viability and a reduction in the frequency of unwanted insertions and deletions in CssDNA-edited cells compared to those edited with LssDNA. Furthermore, CssDNA positively influences CFU plating efficiency relative to LssDNA. These outcomes are consistent across various DNA donor template lengths (from 0.6 to 2.2 kb) inserted at multiple targeted loci. Comparative analysis with the conventional viral DNA template delivery (AAV) using a combination of transcriptomic profiling of edited HSPCs by CITE-Seq coupled with

long-term engraftment capacity assessment in NCG mice, unveiled key transcriptomic and phenotypic distinctions in the efficacy of non-viral versus viral DNA donor templates.

The findings underscore the potential of using non-viral ssDNA delivery in conjunction with TALEN® gene editing for gene insertion in long-term repopulating hematopoietic stem cells (LT-HSCs). The circularization of ssDNA increases gene insertion rates in LT-HSCs and enhances their fitness, thereby facilitating the advancement of next-generation cell therapies. This research marks a crucial step towards enhancing the efficacy of gene therapy and improving patient outcomes.

P0586

Perfect is the enemy of good: Adenine Base Editing to recondition a CF-causing mutation

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Adenine Base Editing is a CRISPR-based system that allows precise nucleotide changes with no double strand breaks. It is highly efficient in both replicating and fully differentiated cells, and it requires the least packaging capacity for delivery, which often makes it preferable to other CRISPR approaches. While adenine base editing can only edit A-T base pairs into G-C base pairs, the primary limitation lies in the conventional focus on achieving perfect edits, rather than exploring alternative beneficial outcomes. Here, we outline an "imperfect" adenine base editing strategy to rescue a nonsense mutation that is responsible for Cystic Fibrosis (CF), a life-shortening genetic disease.

c.1624G>T in exon 12 of *CFTR* is the second most common CF-causing mutation. It is associated with a severe phenotype and is currently untreatable. c.1624G>T creates a TGA premature stop codon, G542X, that annihilates the synthesis of the CFTR anion channel. Adenine base editing cannot restore the wild-type (WT) amino acid, glycine (G), at position 542. However, it can be targeted to the non-coding strand of the TGA (stop, X) codon, to convert ACT to GCT and generate a CGA codon (arginine, R) in the coding strand. This strategy would install G542R, which, albeit not WT, is not listed among CF-causing variants and is known to retain CFTR activity.

We tested this alternative, imperfect, editing approach in patient-derived intestinal organoids, a well-established model of CF. We delivered the base editor NG-ABE8e and a G542X-specific sgRNA as ribonucleoproteins encapsulated in Base Editing engineered Virus-Like Particles (BE-eVLPs). Levels of editing were detected by high-throughput sequencing. Restoration of CFTR function was assessed via Forskolin-Induced Swelling (FIS) and Short Circuit Current (I_{sc}) assays.

Our strategy proved successful and attained an average of ~2% editing efficiency, with no bystander effects, in the total, non-sorted, population of transduced organoids. The newly

generated variant restored CFTR activity to nearly 10% of WT levels, which is considered sufficient to recover a mild phenotype.

This study highlights the potential of Adenine Base Editing to rescue, when it cannot correct, severe disease-causing mutations such as G542X, and emphasises the benefit of exploring alternative, yet robust, approaches when conventional methods fall short. While A-to-Y base editors (AYBEs) have now been developed, that could potentially edit the G542X stop codon (TGA, ACT on the non-coding strand) to the WT glycine (GGA, CCT on the non-coding strand), challenges remain on their PAM requirements. Our focus is now on the development of an NG-AYBE with suitable editing window and the optimisation of the delivery strategy, especially for *in vivo* studies in CF animal models.

P0587

A p38 MAPK-ROS axis fuels proliferation stress and DNA damage during CRISPR/Cas9 gene editing in Hematopoietic Stem and Progenitor Cells

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In hematopoietic stem and progenitor cell (HSPC)-based therapies, *ex vivo* activation and cell cycle progression are prerequisites for sufficient rates of gene correction by homology-driven repair (HDR). We recently discovered that gene editing (GE) by co-delivery of nuclease-induced double strand break (DSB) and corrective DNA templates via AAV6 in cultured HSPCs culminates into a p53-mediated DNA damage response (DDR) activation, affecting their functionality post-transplant. However, whether these detrimental cellular responses might be more exacerbated when GE is coupled with prolonged *ex vivo* culture remains to be established. Here, we show that shortening culture time mitigates the p53-mediated DDR, enhancing the reconstitution capacity of edited HSPCs. However, this results in lower HDR efficiency, rendering *ex vivo* culture necessary yet detrimental. To identify determinants of HSPC dysfunction during *ex vivo* culture, we uncovered a multi-step process initiated by the phosphorylation of the stress responsive p38 MAPK, which generates mitogenic ROS that alter HSPC cell cycle dynamics, ultimately converging in proliferation stress-induced DNA damage accumulation. This precise sequence of events causes functional decline and premature exhaustion of human HSPCs, especially when combined with genetic engineering. Thus, p38 MAPK inhibition before gene editing abrogated culture stress by prolonging the duration of the G1/S transition and mitigating endogenous proliferation-related DNA damage. Of note, p38 inhibitor administration preserved transcriptionally defined primitive HSCs, ultimately endowing edited HSPCs with higher multi-lineage differentiation and superior engraftment success throughout serial transplantation. Importantly, by *in vivo* clonal tracking of edited cells, we discovered that pre-treatment with p38 inhibitor led to a higher clonal output compared to control genetically modified cells in terms of number of both indels (HSPC clones that underwent NHEJ repair) and BARs (HSPC clones that repaired by HDR), pointing to the preservation of the rarer HSC subset responsible for long-term hematopoietic reconstitution of

genetic engineered cells. Finally, p38 inhibition mitigates the genotoxicity risk associated with genetic manipulation, paving the way for more effective and safer HSPC-based gene editing applications.

In conclusion, our study sheds light on the mechanisms potentially contributing to the functional impairment of gene edited HSPCs by providing molecular details on how human HSPCs cope with endogenous stress that may arise during *ex vivo* culture. Our data uncover a p38 MAPK-ROS axis as a novel regulator of cell cycle progression and heightened DNA damage in engineered HSPCs and highlights a strategy based on temporary inhibition of p38 MAPK prior to genetic manipulation to endow edited HSCs with superior long-term and polyclonal repopulating capacity for wider and safer gene therapy applications.

P0588

Testing lipid nanoparticles as delivery tool for genome editing of murine haematopoietic stem and progenitor cells

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Warts, hypogammaglobulinemia, recurrent bacterial infections and neutropenia/leukopenia with myelokathexis (WHIM) syndrome is a rare, combined immunodeficiency disorder caused by heterozygous autosomal dominant mutations in the gene encoding the chemokine receptor CXCR4. The most common mutation (R334X) truncates the intracellular C-tail of the CXCR4 protein, resulting in desensitisation to ligand-mediated internalization and myelokathexis, due to bone marrow leukocyte retention. To date, immunoglobulin supplementation, administration of recombinant G-CSF, low-dose plerixafor, and HPV vaccination are the standard treatments to reduce the risk of infection in WHIM patients, but there is no definitive cure yet.

We have thus developed a novel base editing strategy exploiting ABE8 to correct the WHIM mutation, CXCR4-R334X, in haematopoietic stem and progenitor cells (HSPCs). To circumvent the limited availability of patient cells and preclinically assess the efficacy of the strategy, we are exploiting the murine model, carrying the R334X mutation and recapitulating WHIM disease. To this aim, we are tailoring the BE protocol on murine HSPCs by exploiting lipid nanoparticles (LNPs) as delivery platform.

We first screened several culture conditions and identified one yielding the highest expansion rate of lineage-negative (Lin-) Sca1+cKit+ (LSK) cells from the bone marrow of wild-type (WT) mice. Next, we tested different timing and doses of LNPs for the delivery of GFP mRNA in Lin- cells. We monitored the cell growth, GFP expression and HSPCs composition by flow cytometry at 24, 48, 72 and 96 hours after the LNP-GFP delivery.

Two and three days of culture before LNP-GFP transfection enabled higher cell growth. We achieved more than 90% of GFP expression in LSK cells at 24 hours post-delivery, irrespectively of the timing of LNP-GFP exposure. However, higher GFP expression was observed in LSK cells, at later time points, when LNPs were administered at two or three days of culture. GFP expression was higher in LSK cells than in bulk Lin- cells, likely due to their lower cell cycle rate and/or higher low-density lipoprotein receptor (LDLr) expression. Looking at the phenotypic composition, we observed that our LNP-based protocol preserved the stemness of cells by increasing the frequency of LSK cells over time. In summary, delivering LNP-GFP after two and three days of culture showed the best outcomes in terms of viability, GFP expression and stemness. These preliminary results prove that murine HSPCs are highly permissive to mRNA delivery by LNP transfection *ex vivo*. The next step will be to apply this protocol to HSPCs isolated from WHIM mice and to exploit the LNP-mRNA technology for gene correction of the CXCR4-R334X WHIM mutation by base editing and ultimately prove the efficacy of this approach in the preclinical disease model.

P0589

TERC mediated gene editing for the treatment of autosomal dominant dyskeratosis congenita

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Dyskeratosis congenita (DC) is a rare inherited syndrome mainly associated with bone marrow failure (BMF) and cancer predisposition, and is caused by mutations in genes critical for telomere biology. The clinical presentation of the disease is variable, ranging from hardly detectable, to severe forms causing death in early childhood. Mutations in the RNA component of telomerase gene (*TERC*) have an autosomal dominant (AD) inherited pattern and have been found in 10-20% of DC patients. Currently, allogeneic hematopoietic stem cell (HSC) transplantation is the only curative therapy for the BMF of these patients, although it is still associated with high risks mainly due to the necessity of using genotoxic conditioning. Gene therapy is thus considered an alternative approach to treat BMF in these patients. Aiming to correct *TERC* mutations under conditions that preserve the physiological expression of the corrected gene, we evaluated the efficacy of an *ex vivo* homology-directed repair gene editing approach (HDR-GE) using a designed Cas9/gRNA and AAVs carrying a template donor of *TERC* that harboured a silent mutation in the PAM sequence. The efficacy of HDR was first tested in healthy donor cord blood $CD34^+$ cells ($HD\text{-}CD34^+$). Up to 80% of in vitro cultured human hematopoietic cells and up to 40% of cells from NSG mice transplanted cells exhibited HDR events. Next, we assayed this HDR-GE approach in an AD-DC LCL that harboured the 408C>G *TERC* mutation. In this case, up to 50% of the cells showed corrective HDR events in *TERC*. To assess the efficacy of the gene editing approach in diseases AD-DC HSCs, and given the limited availability of BM samples from AD-DC patients, AD-DC-like HSCs were generated by NHEJ-CRISPR/Cas9 editing in different TERC domains of $HD\text{-}CD34^+$ cells using designed gRNAs. Deleterious *TERC* mutations were found in

20% to 80% of edited CD34+ cells. In these AD-DC-like CD34+ cells, the HDR approach previously used in LCLs reduced the presence of *TERC* mutations up to 25%, with an efficacy that was inversely proportional to the distance between the HDR-inducing cleavage site and the *TERC* domain that harboured the mutated sequence. Due to the reduced clonogenic potential associated to HDR-edited AD-DC like HD-CD34+ cells, we are currently testing additional editing approaches to minimize the toxicity of the procedures. Our results suggest that HDR-mediated gene editing constitutes a feasible approach to correct *TERC* mutations in human HSCs from AD-DC patients, although further efforts are required for the translation of the procedure to the clinics.

P0590

Zinc-Finger Dependent Recombinases for Precision Genome Editing

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Recombinases are promising tools for genome editing due to their ability to manipulate DNA without causing double-strand breaks or relying on cellular DNA repair mechanisms. Tyrosine site-specific recombinases can perform diverse DNA editing reactions, such as excision, integration, inversion, and cassette exchange. By employing directed molecular evolution, the specificity of recombinases can be altered to target specific genomic sequences, allowing for potential therapeutic application. This step-by-step evolution process to modify target site specificity can generate site-specific recombinases capable of genome editing. These unique enzymes can be further optimized for activity through the fusion of recombinases with zinc-finger DNA-binding domains (ZFDs), making them amenable for therapeutic applications. In our study, we systematically optimized parameters of the fusion architecture, including the optimal spacing and orientation of the ZF-binding motif relative to the recombinase recognition sequence and the optimal linker length between the recombinase and ZFD. We developed N- and C-terminal ZF-recombinase fusions that increased activity of the recombinases by up to 10-fold. Moreover, utilizing pentapeptide scanning mutagenesis, we identified optimal insertion points within the recombinase coding sequence for ZFD fusions. The developed insertional ZF–recombinase fusions render recombinase activity dependent on ZFD binding to its target sequence. Thereby these hybrid proteins remain inactive until the ZFD binds to its target near the recombinase recognition sequence, ensuring that recombination occurs only at the intended sites. We applied our approach to the D7 recombinase, developed to correct a large genomic inversion of the F8 gene, which causes severe hemophilia A. We designed ZFDs for sequences flanking the recombinase target site in the endogenous human locus and developed a molecular evolution approach to improve the properties of these ZFDs. The insertional ZF-recombinase fusions demonstrated a four-fold improvement in targeted editing efficiencies and eliminated off-target activity in mammalian cells as assessed *in vitro*. Notably, the ZFD-dependent activity was transferable to recombinases with relaxed specificity, allowing the rapid creation of recombinases capable of recombining multiple target sites in a ZFD-dependent manner. In conclusion, our engineered zinc-finger dependent recombinases represent a new paradigm in genome editing, offering improved precision, efficiency, and programmability. This advancement paves the way for more predictable and versatile DNA editing techniques, with significant implications for both basic research and therapeutic applications.

P0591

Modified single-stranded DNA with truncated Cas12a-target sequences increases gene editing efficiency in primary human T cells

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CRISPR-Cas gene editing has become a widely adopted method for inserting transgenes at precise genomic locations to develop cellular therapies. The efficiency of this editing is influenced by several factors, including the targeted locus, the specific programmable nuclease, and the homology-directed repair (HDR) template. Common HDR templates include plasmids, linear double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA). While ssDNA exhibits reduced toxicity, knock-in rates for larger ssDNA templates are typically low, except when using small single-stranded oligonucleotides. To enhance editing efficiency, researchers have introduced modifications such as adding truncated Cas target sequences (tCTS) to the ends of dsDNA or ssDNA HDR templates. These tCTS are designed to improve template delivery into the cell nucleus by acting as binding sites for the Cas protein, which contains nuclear localization signals. Although *Streptococcus pyogenes* Cas9 (SpCas9) remains the most preferred nuclease, Cas12a Ultra from *Acidaminococcus species BV3L6* (AsCas12a) offers a promising alternative with the potential of expanding the therapeutic genome editing landscape and reducing off-target effects. Since ssDNA with tCTS (ssCTS) has not yet been modified for use with nucleases other than SpCas9, we set out to extend its application to AsCas12a.

Initially, we evaluated whether existing protocols for generating ssCTS using the SpCas9 nuclease would yield comparable results in our experiments. We then explored various AsCas12a binding motifs as end modifications in double-stranded DNA (dsDNA) templates. After optimizing the CTS sequence in dsDNA templates, we investigated the previously reported Mg2+-dependent ssDNAse activity of AsCas12a *in vitro*. By measuring *cis*- and *trans*-cleavage under different Mg2+ concentrations and testing the nuclease in electroporation experiments, our findings indicated that AsCas12a can be highly efficient when used with ssCTS templates. Subsequently, we assessed various parameters concerning the binding of AsCas12a to the CTS, including examining the use of intact versus truncated crRNA complementary sequences and the impact of a buffer sequence. Finally, we evaluated the HDR efficacy and toxicity of various DNA templates with or without CTS at three distinct loci within the T-cell receptor-CD3 complex genes. Non-viral gene editing of T cells with ssCTS and AsCas12a consistently demonstrated improved HDR efficiencies and cell viability at the highest template concentration, regardless of the specific locus or transgene inserted.

In summary, by incorporating CTS to enhance the knock-in efficiency and leveraging the reduced cellular toxicity of ssDNA, ssCTS templates present a compelling alternative to dsDNA-mediated HDR repair in CRISPR-Cas12a gene editing. Additionally, they provide a promising solution to achieving a sufficient number of edited cells without requiring additional purification steps or pharmacological enhancers, as high concentrations can be used without significant toxicity.

P0592

Optimized homologous recombination gene editing in RPL5-like Diamond-Blackfan anemia HSCs

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Diamond-Blackfan anemia (DBA, OMIM #105650) is an inherited bone marrow failure syndrome primarily characterized by erythroblastopenia, congenital abnormalities, and an increased cancer risk. The most frequently mutated genes in DBA are *RPS19* (25%) and *RPL5* (7-11%). A clinically applicable lentiviral-mediated gene therapy approach for RPS19-deficient patients is under development in our lab. However, RPL5 might require a tighter regulation as it interacts directly with MDM2, the master regulator of p53. Therefore, we developed a homologous recombination (HR) gene editing (GE) strategy for RPL5-deficient HSCs, applicable to most mutation types in this gene. This GE approach uses CRISPR/Cas9 system and adeno-associated viral vectors serotype 6 (AAV6) containing a codon-optimized *RPL5*-cDNA sequence (*CoRPL5*).

A delivery optimization of the therapeutic donor was conducted using single-stranded (ssAAV6) and self-complementary (scAAV6) configurations in healthy donor (HD) cord blood (CB) and bone marrow (BM) $CD34^+$ cells at various multiplicities of infection (MOIs). In HD-CB cells, scAAV6 showed higher efficiency and lower toxicity compared to ssAAV6. This facilitated to decrease the MOI by 10-30 times, and was compatible with up to 84% edited alleles using $3x10^3$ genome AAV copies per cell (GC/cell). The transplantation of HD CB-$CD34^+$ gene-edited cells into NBSGW mice demonstrated high engraftment capacity and no impaired differentiation potential following the GE protocol. HR analyses revealed up to 12% edited alleles in human cells engrafting the BM of recipient mice.

To evaluate therapeutic efficacy, RPL5-like cells were generated using CRISPR/Cas9 system on HD CB-$CD34^+$ cells, obtaining 70% *RPL5*-knockout (-KO) indels and resembling the characteristic DBA erythroid defect. Applying our GE protocol in RPL5-like cells showed partial recovery of the DBA phenotypic defect in the erythroid lineage, increasing the erythroid colonies by 2.3-fold and the mature erythroid population ($CD71^-CD235a^+$) by 1.9-fold. HR analysis showed 70% edited alleles 24 hours post-nucleofection. Additionally, a progressive decline in the *RPL5*-KO indel frequency was observed in the control groups, whereas this frequency was maintained in the gene-edited condition. Individual colony analysis revealed that no colonies exhibited biallelic KO of endogenous *RPL5*, except in the gene-edited condition, where up to 50% of colonies were $RPL5^{-/-}$. HR analysis confirmed GE in all $RPL5^{-/-}$ colonies, with 75% showing also biallelic targeting ($CoRPL5^{+/+}$). These results suggest that GE of *RPL5* could have a potential therapeutic benefit in RPL5-deficiency.

To further optimize GE in BM-$CD34^+$ cells from RPL5-DBA patients, an IL1-inhibitor was tested to mitigate the inflammatory effects of the GE protocol in RPL5-like cells. The results showed a 7-fold increase in erythroid colonies and a 1.6-fold increase in growth capacity in the GE+IL1-inhibitor condition compared to GE alone, without altering HR outcomes. These findings suggest that incorporating anti-inflammatory molecules into the GE protocol may significantly reduce acute toxicity, crucial for preventing cell death in RPL5-deficient DBA cells and enabling them to benefit from the therapy.

Overall, our research demonstrates a promising GE strategy for RPL5-deficient HSCs, highlighting the importance of delivery optimization, therapeutic donor configuration, and supportive treatments, like IL1-inhibitor, to mitigate toxicity and improve therapeutic outcomes.

P0593

Preclinical validation of CRISPR/Cas genome editing approaches as advanced therapy for HBB$^{IVSI-110(G>A)}$ thalassemia

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β-Thalassemia, a global single-gene disorder, is caused by deficient β-globin production, with the prevalent mutation HBB$^{IVSI-110(G>A)}$ creating an aberrant intronic splice site. This mutation has a high carrier frequency in Cyprus (76%) and many EU countries (>20%). A mutation-specific gene therapy has been developed using two approaches: a DSB-dependent approach with CRISPR/Cas9 RNA-guided nuclease (IVSI-110 RGN) and a DSB-independent approach with a nearly PAM-less SpG Adenine Base Editor (SpG7 ABE). Both methods disrupt HBB$^{IVSI-110}$ abnormal splicing elements, achieving clinically relevant efficiencies in patient-derived HSCs in vitro. IVSI-110 RGN introduces indels via the non-homologous end joining repair mechanism, while SpG7 ABE uses targeted base (T>C) substitutions.

The project aims to advance these methods by conducting preclinical evaluations of edited cells both in vitro and in vivo using chimeric NBSGW mice, specifically engineered to facilitate human HSC engraftment without irradiation. The primary objective is to confirm the therapy's readiness for clinical trials, focusing on efficacy, safety, and the long-term repopulation (LTR) potential of modified cells. Additionally, the study seeks to compare the mutation-specific approach with a universal therapy targeting the erythroid BCL11A enhancer element for HbF induction (sg1617 RGN), recently FDA-approved as the first CRISPR therapy for sickle cell disease.

RGNs and ABE were delivered via nucleofection to mobilized HBB$^{IVSI-110(G>A)}$ patient-derived HSCs as ribonucleoprotein complexes (RNPs) and in vitro transcribed mRNAs, respectively. The therapeutic potential was evaluated in vitro through induced erythroid differentiation (ED) cultures, assessing correction at DNA (on- and off-targeting, Sanger sequencing), protein (RP-HPLC), and late-stage ED levels (flow cytometry), as well as clonogenic assays for erythroid and myeloid lineage potential. In vivo assessment involved xenotransplantation in NBSGW mice to evaluate LTR potential 16 weeks post-transplantation (flow cytometry).

Overall, both mutation-specific genome editors let to high on-targeting (IVSI-110 RGN 85%; SpG7 ABE (T>C): IVSI-106 and -108: ~40%; -109: ~18%) with undetected off-targeting, and

moderate disrutprion of erythroid BCL11A enhancer element (sg1617 RGN: ~40%). RP-HPLC analysis of the in-vitro ED cultures, showed significant increase of HBB/HBA ratios to normal levels (0.9-1) in IVSI-110 RGN- and SpG7 ABE-genome edited cells and a significant increase of HBG/HBA ratios in the sg1617 RGN edited population (~0.51) relative to UT control (HBB/HBA: ~0.39; HBG/HBA: ~0.27). There was a clear correction of late-stage erythroid differentiation in the mutation-specific edited populations, while genome editing didn't affect erythroid and myeloid lineage potential of HSCs. Analysis of BM chimerism in xenotransplanted NBSGW mice showed high engraftment for all samples (hCD45+: 65% and hCD34+ves: ~6.5%). When comparing genome editing levels between BM bulk inputs and primary recipient BM cells, a 50% reduction was observed in IVSI-110 RGN, while sg1617 RGN showed consistent levels, and SpG7 ABE demonstrated a 20% increase.

Even though, analysis of the biosafety of the genome editing tools is still in progress, the current data indicates ABE SpG7 as the most promising approach for clinical application, since therapeutic levels were achieved while the erythroid and myeloid-lineage and LTR capacity of the edited HSC population was maintained.

P0594

Preclinical In Vitro and Ex Vivo Evaluation of EPI-321, an Investigational Single Dose Epigenome Editing Gene Therapy, Efficacy for Facioscapulohumeral Muscular Dystrophy (FSHD) Treatment

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common types of adult muscular dystrophies with an annual incidence rate of ~ 1 in 10,000, affecting approximately 1 million people globally. With no cure available, current therapeutic strategies only involve managing symptoms to improve overall quality of life. Misexpression of disease-causing protein, DUX4, in muscle leads to slow and progressive muscle degeneration through activation of apoptotic and other downstream pathways. *DUX4* gene is encoded at the distal region *4q35* chromosome from D4Z4 macrosatellite array. In FSHD patients, the D4Z4 macrosatellite array is hypomethylated, leading to stochastic and transient *DUX4* expression, which makes the development of cure challenging.

EPI-321 is an investigational drug product for the treatment of FSHD. It is a single vector AAV*rh74* encoding an ultracompact, dead Cas protein fused to gene-suppressing modulators expressed from a muscle specific promoter, and a gRNA targeting *D4Z4* locus to permanently suppress *DUX4* expression through re-methylation of the D4Z4 locus.

Our pre-clinical studies showed that EPI-321 administration leads to robust and dose-dependent transduction and expression of AAV cargo, consequential suppression of *DUX4* and DUX4-downstream gene expression in ten different FSHD patient-derived immortalized and primary myoblasts *in vitro*, irrespective of the number of D4Z4 repeats, and showed significant anti-apoptotic activity. Mechanistically, EPI-321 showed re-methylation of the D4Z4 target locus leading to suppression of *DUX4* expression. Further, 3D engineered human muscle tissue (3D EMT) using FSHD patient-derived immortalized myoblasts transduced by EPI-321 resulted in

efficient suppression of *DUX4* up to 46 days and demonstrated significant dose dependent improvement in muscle contractility and strength, shown by increased twitch and tetanic force post-treatment.

Taken together, our findings provide robust evidence for EPI-321 as a potential single-administration, “one-and-done”, gene therapy for treating FSHD by permanently suppressing the pathogenic *DUX4* gene through epigenetic silencing.

P0595

AAV-mediated delivery of a novel Cas13 editor achieves specific knockdown of AQP1 and stable reduction of IOP in two different mouse models of glaucoma

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Introduction

Glaucoma is one of the leading causes of adult blindness, affecting over 80 million individuals globally. Elevated intraocular pressure (IOP) is recognized as a significant risk factor in the development of glaucoma, and lowering IOP is the only clinically proven approach to halt or slow the progression of glaucoma. While conventional treatments have demonstrated efficacy in reducing IOP, patient adherence to these interventions remains suboptimal.

Methods

To reduce IOP with a single administration, we have developed a novel AAV-based gene therapy “RM-103” using Cas13-based RNA targeting system (CasRm) to suppress aquaporin 1 (Aqp1) expression and reduce aqueous humor production. To obtain an AAV serotype that can highly specifically infect non-pigmented epithelial cells (NPECs) of the ciliary body, three serotypes (A, B, C) of AAV carrying GFP expression cassette were intracamerally injected into mouse eyes, and the fluorescence intensity was quantified to assess tissue-specific distribution. The potential of RM-103 in treating glaucoma was evaluated in carbomer-induced glaucoma mouse model and DBA/2J model, which is a widely recognized model for spontaneous glaucoma.

Results

The AAV serotype A showed robust EGFP expression and specificity in the NPECs, which was selected. RM-103 administration resulted in over 35% decrease in the Aqp1 mRNA expression in the ciliary body of mice (n=6). Immunofluorescence (IF) assay also indicated a decreased AQP1 expression in the NPECs. In the carbomer-induced model, mice treated with carbomer 4 weeks after a single dose of RM-103 showed 39.3% lower IOP compared to the control group in which mice were treated with control AAV. In the DBA/2J model, the regular monitoring data of IOP revealed a gradual increase curve from 2 to 9 months within the range of 6-11 mmHg. Mice

injected with RM-103 exhibited an obvious IOP reduction after three weeks post-dosing compared to the control group, and the reduction continuously maintained. Throughout this period, the maximum decrease in IOP was 26.7%. **Conclusions** RM-103 represents a potent gene therapy for treating glaucoma, which can efficiently and continuously reduce IOP in mouse models through a one-time treatment. These results indicated that CasRm-based RNA targeting system is a novel potential approach in gene therapy for glaucoma.

P0596

Base editing mediated disruption of *RASA2* improves multiplex gene edited CAR-T cells

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Adoptive transfer of T cell receptor (TCR) and chimeric antigen receptor (CAR) redirected T cells represents a promising treatment modality for hematologic malignancies. However, expanding cellular therapies to broader patient populations comes with several challenges regarding efficacy, persistence, and product safety. Next-generation genome editing technologies are versatile tools that allow precise modification or elimination of genes. Hence, the ability to modify cellular signalling pathways is a powerful tool in augmenting cellular therapies to treat complex diseases.

Previous preclinical studies have suggested that *RASA2* is a promising target to enhance TCR- and CAR-redirected T cell therapies by CRISPR-Cas9 mediated knock-out (KO). The *RASA2* gene is involved in controlling cellular activation, proliferation and differentiation in human T cells. Consequently, it´s depletion enhances cytokine production and anti-tumor efficacy of the engineered T cells. However, conventional Cas9-mediated gene editing induces DNA double-strand breaks (DSB), which could lead to unwanted on-target aberrations and significant genotoxicity, especially when multiplexed with other edits. Base editing tools have emerged that allow for the site-specific deamination of adenine (ABE) or cytosine (CBE) without relying on the generation of DSBs. Introducing premature stop codons or altering splice sites are effective strategies for targeted gene silencing.

Here, we identified a highly efficient and DSB-free strategy to disrupt *RASA2* splice sites utilizing an ABE in primary human T Cells. We evaluated three different guide RNAs (gRNA) by Sanger sequencing to determine the base editing efficiency. The preferred gRNA demonstrated >90% A to G conversions in the targeting window. Subsequently, the different base-edited T cells were assessed functionally on account of their cytokine production profile after activation. In line with previous results for conventional Cas9-mediated KO as well as from base editing mutagenesis screens, we observed increased TCR-dependent Type 1 cytokine production in both CD4+ and CD8+ T cells after base editing of *RASA2* (*RASA2* BE). Highly efficient *RASA2* BE was successfully combined with AsCas12a-mediated knock-in of a CD19-CAR at the *TRAC* locus in a single electroporation. When co-culturing the CAR T cells with a CD19+ leukemia cell line, we detected an increase in IL-2 production in the CD8+ *RASA2* BE CAR T cells as well as a slight improvement in Type 1 cytokine production in both CD8+ and CD4+ *RASA2* BE CAR T cell subsets. We are currently evaluating the suitability of the *RASA2* BE strategy to enhance the cytotoxic capacity and cytokine secretion with other CAR constructs and target cells. Further, we are developing assays to quantify chromosomal translocations between the *TRAC* and *RASA2* loci.

After careful evaluation of genotoxic events in multiplex edited cells, *RASA2* BE may allow optimizing the performance of TCR- and CAR-redirected T cell products for diverse treatments of cancer and autoimmune diseases.

P0597

Precise correction of the sickle cell disease mutation by DNA repair manipulation and genome editing in hematopoietic stem cells

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The CRISPR/Cas9 system allows for precise correction of genetic mutations that lead to severe genetic diseases, such as sickle cell disease (SCD). In this project we explore gene editing approaches relying on Cas9 and ssODN DNA donors for precise repair of the SCD mutation, through homology-directed repair (HDR). HDR is active in proliferating cells and, therefore, occurs at low rates in quiescent hematopoietic stem/progenitor cells (HSPCs), the target cell population for gene therapy of SCD. Mutagenic DNA repair pathways, such as non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ), are activated upon Cas9-induced DNA double-strand formation and lead to the generation of undesired insertions and deletions (InDels).

Here, we screened single guide RNAs (sgRNA) and ssODNs with Cas9 nucleases and identified efficient combinations that allowed high knock-in efficiencies in erythroid cell lines, as assessed by next generation sequencing (NGS). DNA repair profiling revealed the presence of NHEJ and MMEJ-mediated InDels. To circumvent this, we used inhibitors of key enzymes of NHEJ and MMEJ repair (2iHDR), thereby enhancing HDR-mediated integration efficiencies and editing precision. We further optimized the editing outcome by increasing the Cas9, sgRNA, and DNA donor concentrations and by implementing Cas9 targeting sites in the DNA donor – known to increase knock-in efficiencies.

To apply 2iHDR in HSPCs, we initially performed drug titrations and identified optimal drug concentrations that allowed enhancement of precise genome editing and minimal effects on HSPC fitness, measured by a series of assays (cell counting, flow cytometry analysis of necrosis and apoptosis, expansion rate and CFC assay). We then combined the optimized genome editing strategies with optimal 2iHDR in HSPCs, allowing therapeutically relevant levels of precise SCD correction and minimal InDels. FACS-sorting of HSPC subpopulations, upon editing and drug treatment, showed that our strategy works similarly in HSC-enriched *versus* progenitor subpopulations without affecting their viability and clonogenic potential.

Moreover, we assessed the safety of our strategy in terms of off-target activity and genomic rearrangements. NGS showed that 2iHDR did not increase the off-target activity of SpCas9. We also used PsCas9 – a high-fidelity enzyme that is equally efficient to SpCas9. Indeed, no off-target activity was observed with PsCas9 when targeting the SCD mutation, while on-target editing was higher for PsCas9 than SpCas9. DdPCR confirmed the safety of PsCas9 and 2iHDR, with regards

to genomic rearrangements. Additionally, we performed RNA-seq analysis to evaluate the global effect of 2iHDR in HSPCs, and we used barcoded ssODNs to track the clonality of treated HSPCs, confirming no effect of 2iHDR on the HSPC clonal pool.

To conclude, we provided proof for the safety and efficacy of 2iHDR-mediated precise correction of the SCD mutation in HSPCs. *In vivo* xenotransplantation of treated HSPCs in immunodeficient mice is ongoing to evaluate the efficacy of our approach in repopulating HSCs. Importantly, 2iHDR could be applied to correct other genetic mutations that are not amenable to alternative genome editing tools, such as base or prime editing. This proof-of-concept work will enable the pre-clinical and clinical development of 2iHDR HSPCs for the therapy of SCD.

P0598

Development of a comprehensive framework for assessing the genomic safety profile of RNA Gene Writers

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RNA Gene Writers are unique and engineerable constructs that leverage all-RNA components and harness the mechanism of target-primed reverse transcription, or TPRT, to introduce a broad range of edits to the genome, from inserting a transgene or exon to introducing single nucleotide changes. RNA Gene Writers are designed to provide an attractive toolkit for the treatment of a wide variety of genetic diseases.

Here we will focus on the genomic safety assessment of RNA Gene Writers developed to correct mutations leading to various monogenic diseases including classic phenylketonuria (cPKU) and alpha-1 antitrypsin deficiency (AATD). We developed an RNA Gene Writer designed to target the R408W mutation in the *PAH* gene causing cPKU and observed an average of 47% of alleles being corrected in the liver of a novel humanized PKU R408W mouse model after a single dose. Similarly, we developed an RNA Gene Writer that achieved an average of 49% correction of the E342K mutation in the gene responsible for AATD in the liver of a mouse model of disease. One of the key needs for characterizing any new genome engineering system advancing through preclinical development is the building of a comprehensive data package assessing genomic safety.

Here we present a broad framework for assessing the genomic safety profile of our RNA Gene Writers that includes a comprehensive and unbiased off-target discovery platform followed by deep-sequencing-based high through-put validation studies in the cell or tissue types of interest. In addition, we assess structural changes to the genome in the context of RNA Gene Writers developed for correcting the PKU R408W mutation in the liver. By using this comprehensive methodology of orthogonal assessments of genotoxicity we characterized the genomic safety profile of our RNA Gene Writers that supports the technology's potential to generate a new class of highly potent and safe genomic medicines.

P0599

Miniaturized Genome Editing Tools from the Microbiome: Unveiling the Potential of IscB and TnpB proteins

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IscBs and TnpBs, Cas9 and Cas12 ancestors, are emerging genome editing tools that offer exciting possibilities with their miniaturized size and editing abilities. Naturally encoded within prokaryotic transposable elements, these proteins play a dual role: facilitating the transposition process and promoting transposon survival. Analogously to CRISPR/Cas systems, they work as a ribonucleic complex encompassing a protein and a guide RNA, namely wRNA, to introduce a double strand break on a DNA target. This occurs upon Target Adjacent Motif (TAM) recognition and guide hybridisation with the target sequence. IscBs and TnpBs are c.a. 400 amino acids long and their wRNA is c.a. 200-400 nucleotides, making them exceptionally compatible with delivery tools including all-in-one AAV vectors in the form of the nuclease, base or epigenome editors.

Considering the unique properties outlined above, our project aims to characterize novel IscBs and TnpBs within the human microbiome. Bioinformatic analysis of an extensive dataset of metagenome assemblies generated a set of IscB proteins with high copy number and a good wRNA folding prediction, plus a pool of TnpB proteins with conserved amino acids at relevant sites, low identity with literature TnpBs and a high copy number as well. Owing to their original function, IscBs and TnpBs TAM sequences exhibit minimal variation, hence proteins with heterogeneous predicted TAMs were also included. TAMs were confirmed via an *in-vitro* assay and activity was preliminarily assessed via a round of screening on GFP. The best selected nucleases were also tested towards genomic loci. Overall, we obtained selected IscBs and TnpBs candidates that are currently under development through molecular engineering for optimization as editor tools as nuclease and second-generation base and epigenome editors.

With our exploratory efforts we are focusing on uncovering promising IscB and TnpB candidates with the ultimate goal of advancing the top performers to expand the genome editing toolbox more compatible with delivery and wider targeting range in the genome.

P0600

Reverting the F508del-CFTR defect in cystic fibrosis with an efficient and safe genome editing approach

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Cystic Fibrosis (CF) is the most common life-shortening autosomal recessive disease in the Caucasian population (incidence of 1:2500 individuals).

Even though pharmacological therapies can improve the quality and life expectancy of patients, still a definitive cure is missing.

CF is caused by mutations in the CFTR gene (cystic fibrosis transmembrane conductance regulator) which encodes for a transmembrane channel with a crucial role in the secretion of chloride and bicarbonate. In CF, the impairment of CFTR affects different organs and the main cause of morbidity is the development of a progressive obstructive lung disease.
More than 700 mutations in the CFTR gene are known to cause CF. The most common is the F508del (present in 80% of patients), a deletion of 3 nucleotides that causes defects in the folding of CFTR protein, leading to a premature degradation of CFTR by the ubiquitin-proteasome system. Attempts to correct F508del using CRISPR-Cas9 technology have been made over the years, however, the strategies used so far are limited in efficiency. Furthermore, most of them are based on double-strand breaks (DSBs), thus increasing the risk of genotoxicity determined by large genomic rearrangements.

Here we used CRISPR-base editors, inducing single-base substitution without generating DSBs, to insert secondary mutations in the F508del locus in order to neutralize the genetic defect. This approach was inspired by previous studies showing that secondary mutations in cis with F508del can stimulate proper folding and function of CFTR protein; these mutations are called "revertants".

Our results show that a specific combination of base editor (ABE or CBE) and multiplexed sgRNAs can efficiently introduce revertants in the CFTR gene of cell lines, obtaining up to 60% of base conversion. The efficacy of this strategy was validated in cell lines and in primary cells derived from F508del patients. In both experimental models we measured a functional recovery of CFTR activity near wild-type level. Finally, the efficacy of combinatorial treatment with pharmacological treatments was evaluated.

Overall, we demonstrated that our base editing approach to correct the F508del is an efficient and safe strategy paving the way for the development of a possible new therapy that offers a definitive cure to CF patients.

P0601

Programmable genomic integration (PGI), a novel gene editing approach for Phenylketonuria (PKU)

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Phenylketonuria (PKU) is a rare autosomal recessive metabolic disorder caused by mutations in the phenylalanine hydroxylase (PAH) gene, which is responsible for conversion of phenylalanine (Phe) to tyrosine (Tyr). Mutations in the PAH gene result in accumulation of Phe and reduction of Tyr concentration in the blood and brain, resulting in abnormal brain development in children and psychiatric and cognitive problems in adulthood. The current treatment for PKU is a strict diet that limits Phe intake, but this is costly and difficult to maintain and does not prevent all neurological complications. Gene editing offers a potential permanent cure and animal models

suggest that just 10% of hepatocyte correction is required to rescue disease phenotype, but the numerous mutations in PAH (~400) present significant challenges for targeted small gene editing strategies. Instead, Tome has developed integrase-mediated programmable genomic integration (I-PGI) as a novel treatment for the vast majority (>95%) of PKU patients by inserting a functional copy of the PAH gene into the endogenous locus.

In I-PGI, Cas9 nickase is coupled with a writing enzyme to place an integrase recognition site ('beacon') at a user defined location in the genomic locus of interest. After beacon placement, an integrase and DNA cargo containing the other recognition site result in targeted unidirectional gene insertion without dependence on double stranded breaks (DSBs) or homology directed repair (HDR). Through sequential optimization of guide RNA, editing enzymes, delivery systems, and cargo, we demonstrate that I-PGI can efficiently and precisely insert the PAH gene into the PAH locus in both *in vitro* and *in vivo* models. *In vitro*, we have achieved over therapeutically relevant edit levels (>35% gene insertion) in primary human hepatocytes (PHH). *In vivo*, though continuing to advance our technology, we have already achieved potentially curative levels of gene insertion (up to 13%) in non-human primates (NHPs). Additionally, analysis of gene expression in edited NHP liver shows that allelic correction significantly underestimates actual hepatocyte correction by at least a factor of 2, further supporting the efficiency of I-PGI treatment.

To summarize, through improvements in every component of our technology, we have achieved highly efficient I-PGI in clinically relevant primary human cells and potentially curative levels of editing in NHPs. This PoC data supports a new therapeutic approach for PKU, in which a healthy copy of the PAH gene is inserted into its endogenous location. Our approach has the advantages of keeping the PAH gene under endogenous regulation, having a single product as the treatment for most patients (mutation agnostic), and the potential for pediatric treatment as the integrated gene will grow with the patient.

P0602

CRISPR/Cas9-induced genomic alterations and AAV-transgene targeted integration in HSPC through the lens of Long Read-sequencing

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CRISPR/Cas9 technology has revolutionized our ability of genome editing and is widely used as a tool for gene therapy. However, recent studies show it can result into extensive on-target genomic damage (extended deletions, chromosomal loss, translocations) in edited human cell lines, primary cells or human embryos. In addition, little is yet known about CRISPR mediated targeted integration outcomes in primary cells.

Here, we carried out a detailed analysis of the genomic rearrangements occurring upon CRISPR/Cas9-based gene editing, alone or in combination with targeted integration of a therapeutic viral vector in cell lines and clinically relevant Hematopoietic Stem/Progenitor Cells (HSPCs). To that end, we performed long read sequencing (LRS) of a targeted genomic region of interest (ROI), using the Oxford Nanopore Technology (ONT) and a Cas9-based enrichment procedure, avoiding PCR amplification biases.

We targeted three genomic loci: AAVS1 safe harbour, α-globin (which we have previously characterized as a safe harbour for editing and targeted integration in human HSPCs) and β-globin loci. We used Cas9-gRNA ribonucleoprotein complexes alone or with an adeno-associated virus (AAV) encoding for a transgene, flanked by homology arms for homologous recombination. We performed Cas9-enrichment of a ~15kb ROI to recover most possible editing events with statistical significance (~100X coverage) and we established a bioinformatical pipeline to analyse the generated data and explore the structural variations as well as DNA methylation profile.

We showed that large unintended deletions (> 100bp) at the gRNA cut site occur at similar frequencies in HSPCs and in K562/HUDEP-2 cells (~20%) at all targeted loci. The size and frequency of these deletions is decreased in presence of an AAV template. We observed that AAV integration efficiency is cell type dependent, with higher efficiency in HSPCs (>50%) rather than K562/HUDEP-2 (~20%), similar for the three studied loci. Most of the integrations are HDR mediated, with some cases of AAV ITRs on-target-trapping detected, likely due to non-homologous end joining (NHEJ). Surprisingly, we revealed the existence of complex rearrangements of integrated AAV genomes, with concatemers accounting for 20% of the AAV integrations. ITRs are also detected in AAV concatemers integration, on the junctions of the copies. We performed LRS on the plasmid preparations and AAV productions and did not observe such rearrangements, suggesting that these events occur within the cell nucleus. Furthermore, local genome methylation pattern is not affected upon gene editing and AAV integration.

To increase AAV HDR integration while reducing the NHEJ integration, we assessed the effect of NHEJ DNA repair inhibitor. We observed that NHEJ inhibition results in the increase of HDR-based targeted integration (2-3fold) and less AAV concatenation. However, it also increases the size and frequency of large deletions (2fold).

We confirmed our results by independent approaches as InDel analysis for editing efficiency, ddPCR and FACS for the analysis of transgene targeted integration and expression.

Our findings provide a clear understanding of the on-target genomic alterations occurring upon CRISPR editing and targeted integration of AAV vectors in the HSPCs. This knowledge is of crucial importance for future clinical applications of the CRISPR gene editing tool.

P0603

Targeted Knock-In at the Human TTR Locus Using Type V Nucleases

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Many genetic diseases, while caused by a single gene, have large numbers of mutations distributed across the length of the gene. Repairing a single mutation or even multiple mutations localized to a small region will leave many patients untreated in the majority of monogenic diseases. Ectopic expression, such as AAV gene replacement, has been used in some cases, but use is limited to recessive disorders where the gene is below the packaging capacity of AAV. Furthermore, ectopic expression often results in expression at an inappropriate level or in the wrong tissue and expression may wane over time. To overcome these limitations, we are developing methods to insert large pieces of DNA, exons to full genes, in the endogenous site of the genome where they will be under physiological control. Specifically, we are delivering

nucleases targeted to the genomic locus and DNA transgene donors and harnessing endogenous DNA repair mechanisms, facilitating precise insertion of the entire coding sequence which should stably correct any mutation.

To test the feasibility of using type V nucleases for whole gene insertion at a therapeutically relevant locus, we focused on the transthyretin (TTR) locus. Transthyretin amyloidosis (ATTR) disease is caused by buildup of TTR amyloids in peripheral nerves and other organs. In its familial form, ATTR is due to missense mutations in the TTR gene leading to an unstable protein that aggregates after cellular secretion. We sought to perform on-target knock-in of wild-type TTR sequence downstream of the endogenous promoter to interrupt the disease-causing TTR V30M mutant in a humanized mouse model of familial ATTR disease and install a TTR variant, T119M, which has been shown to stabilize TTR and potentially compensate for residual expression of TTR V30M.

We assessed several knock-in methods in vitro, in both dividing and non-dividing cell types, to optimize our approach for high efficiency and precision. We found that type V nucleases demonstrate equivalent or better knock-in efficiency compared to type II nucleases, in both HEK293 and non-dividing iPSC-derived hepatocytes. We found that knock-in efficiency correlated with increasing homology arm length, and that donors designed to take advantage of homology-mediated end-joining (HMEJ) pathways led to a marked increase in efficiency, especially in non-dividing cells. Compared to other knock-in donor designs, HMEJ donors also demonstrated the highest rate of precise integration in mouse liver. Finally, using a combination of LNP and AAV delivery, a knock-in system containing the ABR004 type V nuclease and an HMEJ donor achieved 10% in vivo mouse liver integration of a corrective donor targeting the humanized TTR locus. Taken together, these data support the further application of Arbor's diverse toolbox of nucleases toward treating monogenic diseases via targeted whole gene knock-in.

P0605

Prime editing functionally corrects rare and common cystic fibrosis-causing mutations in patient-derived organoids and airway epithelia

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Being a severe and monogenic disease, gene therapy for cystic fibrosis (CF) has been pursued since the early 90's and focused on a gene addition approach. Gene correction on the other hand, allows to restore mutations in patients' chromosomes, thereby preserving endogenous gene expression and regulation and possibly providing a permanent cure. The last three years excited the gene editing fields with CRISPR trials for a handful of genetic disorders, leading to a first approved CRISPR therapy and promising data from pioneering in vivo trials with Cas9 or base editing approaches. We hypothesized that prime editing (PE), one of the most recent gene editing technologies, could be leveraged to correct any CF-causing mutation, including rare and common variants L227R (c.680 T>G) and N1303K (c.3909 C>G) in a permanent and precise manner.

To obtain optimal PE strategies for both mutations, we screened over >100 prime editing approaches in *CFTR*-cDNA engineered HEK293T cells. Gene editing in HEK and 16HBE epithelial cells was verified at protein level by evaluating restored CFTR glycosylation (western blot), plasma membrane (PM) localisation (confocal microscopy/flow cytometry) and ion channel function (HS-YFP quenching). To validate our approach in translational models for CF, we delivered the PE approaches to patient-derived rectal organoids and human nasal epithelial via lentiviral vectors. In organoids, forskolin-induced swelling (FIS) was used to probe for functional *CFTR* correction. Results obtained in patient-derived organoids were validated in human nasal epithelial (HNE) cell cultures differentiated at air-liquid interface, followed by Ussing short circuit electrophysiology. To facilitate faster screening of future gene editing or gene addition strategies, we built a machine learning analysis tool (DETECTOR) capable of rapid and sensitive analysis of functional correction in patient-derived organoid models.

Screening in *CFTR*-cDNA engineered HEK293T delivered guide combinations allowing >25% and >40% correction of L227R and N1303K mutations respectively. Correction on genomic level was associated with restored CFTR glycosylation (band C on western blot), plasma-membrane localisation (confocal microscopy) and restored ion channel function (HS-YFP quenching). Results obtained in HEK293T and 16HBEge cell lines were next confirmed in patient-derived rectal organoids and differentiated airway epithelia. With the sensitive analysis enabled by DETECTOR, we were able to accurately dissect the effect of each PE component on functional rescue and screened enhancements directly in a relevant primary system.

In each model genomic correction of the *CFTR* locus was associated with clear functional correction (up to 80% in patient-derived organoids and 30% in airway epithelial cells). Interestingly, the functional rescue observed in the differentiated HNE monolayers was supported by a mere 1.5% correction on DNA level, indicating that precise correction in a small subset of basal cells could support clinically relevant CFTR currents, delivering a hopeful perspective for future CF gene editing therapies.

Our results show that L227R and N1303K, both severe CF-causing mutations, can be precisely, safely and functionally corrected by prime editing in primary cell models for CF. Ongoing work focuses on tailoring a translational delivery vehicle for the gene editing machinery.

P0606

Efficient correction of a prevalent *COL7A1* mutation via prime editing in recessive dystrophic epidermolysis bullosa

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Prime Editing (PE) is an advanced CRISPR/Cas9-based gene editing technique. It allows for traceless correction of disease-causing mutations without the need for double-strand breaks

(DSB). Via a combination of a Cas9 nickase (Cas9n) and a reverse transcriptase (RT), PE is able to insert any desired sequence at the target site. Epidermolysis bullosa (EB) is a rare, monogenetic skin disease. Due to extremely fragile skin, patients suffer from huge wounds and blisters all over their body. Recessive dystrophic EB (RDEB), one of the most severe forms of EB, originates from mutations in *COL7A1* and is often associated with early onset of squamous cell carcinoma (SCC). In this work we aim for a PE-based correction of a prevalent mutation (c.425A>G) in exon 3 of *COL7A1* that leads to complete absence of type VII collagen (C7).

Isolated RDEB patient keratinocytes were nucleofected with *in vitro*-transcribed (IVT) PE mRNA as well as synthetic prime editing gRNAs (pegRNA) and nicking gRNAs targeting the mutation. Subsequently, cells were analysed via immunofluorescence (IF) staining, digital droplet PCR (ddPCR), flow cytometry, Western blot and next-generation sequencing (NGS).

Our analysis revealed a C7 restoration in 80-90% of treated RDEB keratinocytes with around 80% of tracelessly corrected alleles. Protein levels were comparable to those of healthy keratinocyte donors. Furthermore, NGS of *in silico*-predicted off-target sites showed no signs of undesired PE-induced genomic modifications.

In this project, we were able to confirm that PE is able to combine high correction efficiencies with uniform repair pattern. In addition, our analysis indicates the increased safety of this technique when compared to classical CRISPR/Cas9 strategies. We therefore consider it as a highly promising gene editing technique for EB and other monogenetic diseases.

P0607

Mobilization-based non-genotoxic conditioning for the engraftment of gene edited hematopoietic stem cells in the non-human primate animal model

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In conventional autologous hematopoietic stem and progenitor cell (HSPC) gene therapy, patients undergo mobilization and harvesting of HSPCs- These cells are then genetically corrected *ex vivo* and reinfused in patients after partially or fully myeloablative conditioning to create space in the bone marrow (BM) for the corrected cells. However, the chemotherapy and radiotherapy-based conditioning procedures currently used in clinical settings result in severe acute and long-term side effects, posing significant risks to patients.

To overcome these limitations and progress towards a chemotherapy-free, non-genotoxic conditioning regimen, we previously demonstrated that HSPC mobilization using granulocyte-colony stimulating factor (G-CSF), AMD3100, and BIO5192 effectively depletes the BM niche, making space available and thus creating a window of opportunity for the engraftment of *ex vivo*-cultured cells in a human hematochimeric mouse model. Furthermore, we showed that the transient overexpression of the CXCR4 homing receptor using an improved mRNA *in vitro* transcription platform is compatible with gene transfer and CRISPR/Cas9-based homology-mediated gene editing. This approach confers a competitive engraftment advantage to the transplanted cells over the mobilized recipient cells, allowing an engraftment of corrected cells

that represents up to 30% of the human graft, which is a clinically relevant therapeutic threshold for many primary immunodeficiencies.

While the hematochimeric mouse model provides valuable insights, it may not fully replicate the complex interactions between human HSPCs and the BM niche. Therefore, testing and validation of our conditioning and engraftment strategy in non-human primates (NHPs) are necessary before considering clinical translation. Our findings in the NHP model are consistent with observations in human cells, indicating that CXCR4 expression is successfully rescued during *ex vivo* culture of NHP HSPCs harvested by G-CSF and AMD3100-mediated mobilization. Moreover, the transient overexpression of CXCR4 by mRNA delivery significantly increases SDF-1 dependent migration efficiency in *in vitro* assays. In addition, we demonstrate that adenine base editing at the clinically relevant CD33 locus can be multiplexed with CXCR4 overexpression without compromising the efficiency of either procedure. This combination confers a migration advantage to the edited cells, enhancing their potential for successful engraftment, and opening the possibility to select edited cells by immunotherapy with anti-CD33 immunotoxins.

To facilitate clinical translation, we are currently exploring two different mobilization protocols. The first protocol mimics the approach used in the mouse model, involving a short, single-cycle mobilization where cells are collected, edited, and immediately reinfused. The second strategy involves mobilizing and collecting cells editing and storing them for quality checks and off-target editing analyses. Corrected cells are reinfused after a second mobilization cycle, which serves as a conditioning regimen.

Successful validation of our non-genotoxic conditioning strategy in the NHP model has the potential to pave the way for broader and safer clinical applications of HSPC gene therapy. By leveraging HSPC mobilization and transient CXCR4 overexpression, we aim to minimize the toxicity associated with current conditioning procedures, ultimately offering a promising alternative for patients requiring gene therapy.

P0608

Hematopoietic stem and progenitor cell gene editing for Congenital Dyserythropoietic Anemia type II

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Congenital dyserythropoietic anemia type II (CDAII) is an inherited anemia that affects the development of the erythroid lineage. CDAII patients suffer from ineffective erythropoiesis, hemolysis, iron overload, and the presence of binucleated erythroblasts within the bone marrow. CDAII is caused by mutations in the *SEC23B* gene, with more than one hundred pathological variants described throughout the gene. Allogeneic hematopoietic stem cell transplant (HSCT) represents the only curative option for this disease, but it implies serious side effects. Autologous HSCT of genetically corrected cells would mean a definitive treatment for CDAII.

We have explored a universal gene editing approach to correct all CDAII patients based on the knock-in of the wtSEC23B cDNA into the *SEC23B* locus in hematopoietic stem and progenitor cells (HSPCs). We designed a sgRNA targeting the region immediately upstream of the second

exon of the *SEC23B* gene. This sgRNA exhibited a high on-target activity in human HSPCs and was found to be relatively safe/precise in GUIDE-Seq and CAST-Seq analyses. Subsequently, we constructed two homology-directed repair (HDR) donors as recombinant adeno-associated vectors (rAAV). First HDR donor carried the tGFP cDNA as a reporter to insert it into the *SEC23B* locus and express it under the endogenous SEC23B regulation. The second HDR donor contained a therapeutic wtSEC23B cDNA to be inserted at the same genomic position. After nucleofecting human HSPCs with the sgRNA ribonucleoprotein complex and transducing them with either of the HDR donors, up to 70% gene-edited HSPCs were obtained.

When we performed *in vitro* erythroid differentiation liquid cultures, we observed a reduction in the expression of SEC23B protein in gene-edited HSPCs with the tGFP HDR donor in comparison to unmodified or gene-edited HSPCs with the wtSEC23B HDR donor. We also identified a decrease in the erythroid progenitor frequency and erythroid cell production derived *in vitro* from tGFP gene-edited HSPCs compared to unmodified or wtSEC23B gene-edited HSPCs. Moreover, frequency of tGFP gene-edited cells shrank in the last steps of the erythroid differentiation. At the end of the *in vitro* erythroid differentiation, the frequency of bi/multinucleated erythroid cells tended to increase in tGFP gene-edited cells, suggesting a knocking-out effect due to the tEGFP integration in the *SEC23B* locus. However, this frequency of bi/multinucleated erythroid cells derived from wtSEC23B gene-edited HPSCs was similar to the controls.

We then transplanted tGFP or wtSEC23B gene-edited HSPCs into immunodeficient NBSGW mice. Although we observed a reduction in human engraftment in animals transplanted with gene-edited HSPC, up to 60% gene-edited cells were identified within the human hematopoietic population. Like the *in vitro* erythroid differentiation, the tGFP gene editing frequency diminished in the later stages of the human erythropoiesis *in vivo*. However, wtSEC23 gene editing percentage was similar throughout human erythropoiesis in the NBSGW bone marrow.

In summary, we have developed an effective gene editing approach in HSPCs to insert wtSEC23B cDNA into the SEC23B locus allowing HSPC engraftment and erythroid differentiation. We think that our approach could be considered as a potential gene therapy for CDAII.

P0609

Characterising designer nuclease activity and DNA repair mechanisms with MEGA dPCR and high-resolution CAST-Seq

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Undesired repair outcomes such as indels via the non-homologous end-joining (NHEJ) pathways can compromise the efficacy of therapeutic gene editing strategies using designer nucleases and exogenous donor templates. To mitigate sub-optimal integration efficiency, compounds that inhibit DNA end-joining repair pathways have been harnessed to direct cells to preferentially repair double-strand breaks (DSBs) via the homology-directed repair (HDR) pathway. However, the impact of designer nucleases combined with systemic repair inhibition on repair products generated from off-target activity has not been thoroughly investigated. Additionally, the dynamics of nuclease-induced DSBs and resolution are poorly characterised, partly due to the lack of a single tool capable of providing an unbiased and broad overview of designer nuclease-induced mutations. We addressed this by developing Multipurpose Editing and Genotoxicity

Assessment (MEGA), a novel diagnostic ensemble of multiplexed assays utilising digital PCR (dPCR), which provides a comprehensive characterisation of DSBs and other mutations at both on- and known off-target sites. To quantify chromosomal aberrations, we performed MEGA dPCR and CAST-Seq on CRISPR-Cas9-edited haematopoietic stem and progenitor cells (HSPCs) cultured with an end-joining repair inhibitor cocktail (ART558 and AZD7648). To our surprise, in repair-inhibited cells, MEGA revealed up to 60% of alleles harboured DSBs at three known off-target loci, of which 1.6% of the total alleles resolved as a translocation, up from 0.24% in the absence of repair inhibitors. Additionally, by performing CAST-Seq on repair-inhibited cells, we observed a 15-fold increase in the number of unique translocation events detected, thereby identifying novel off-target nuclease activity that would otherwise remain concealed. Our findings reiterate that careful strategy design should be at the forefront of designer nuclease-based gene therapies. Seeking to adopt repair-inhibiting compounds may improve integration efficacy, but at the cost of genome destabilisation and increasing the risk of generating genotoxic by-products. We propose that utilising DSB repair-inhibiting compounds necessitates a careful specificity evaluation for potential clinical use but nonetheless has value for enhancing the sensitivity of techniques that detect designer nuclease-mediated mutations and characterising DSB generation and repair dynamics.

P0610

Prime Editors precisely correct pathogenic mutations in *RHO* and *USH2A* associated Retinitis Pigmentosa and prevent retinal degeneration

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Retinitis Pigmentosa (RP) is a bilateral and progressive group of retinal diseases characterized by primary loss of rod photoreceptors followed by secondary loss of cone photoreceptors and degeneration of the retinal pigment epithelium (RPE). RP, affects ~200,000 in the USA and EU, is a prevalent cause of visual disability and blindness, and lacks treatments that effectively halt or slow RP disease progression. Prime Editing is a next generation gene editing technology that can precisely correct more than 90% of all pathogenic human mutations without the need for double strand breaks (DSBs) and with minimal byproducts at the edit site and minimal off-target activity.

Leveraging the knowledge and experience previously established in the ophthalmology field for effective gene delivery and good safety profile of AAV vectors, Prime Medicine has developed a Dual-AAV platform to deliver Prime Editing components to photoreceptors in the retina via subretinal injection. Our therapeutic approach utilizes Dual-AAV Prime Editors (PEs) to precisely correct prevalent disease-causing mutations in *RHO* and *USH2A*, which are the most recurrently mutated genes causing RP.

Comprehensive high throughput screening led to the identification of potent Prime Editing components to precisely correct the most prevalent mutations in *RHO*, including p.P23H, p.V345L and p.P347L. We are developing two Dual-AAV PEs, one to address *RHO* p.P23 mutations and another to precisely correct 18 mutations in a mutational hotspot that includes *RHO* p.V345L and p.P347L. Performance of the Dual-AAV PEs was initially assessed in mutant or patient cell assays followed by validation in human retinal explants, where we observed editing efficiencies as high

as 40%. To evaluate gene correction *in vivo*, humanized mouse models were generated in which the mouse *Rho* gene was replaced with a mutant human *RHO* gene. After Dual-AAV PEs were injected subretinally, precise correction of mutations, *RHO* p.P23H or p.V345L/p.P347L, was detected in >60% of photoreceptors within the transduced area, which was associated with preservation of the photoreceptors. Untransduced area show rapid photoreceptor degeneration.

Leveraging the modular Dual-AAV delivery platform established for *RHO* Prime Editing, we selected PE components to correct the most prevalent RP-causing mutations in *USH2A*, including p.C759F (c.2276 G>T) and p.E767fs (c.2299delG). In *USH2A* humanized mice, precise correction of p.E767fs was detected in >60% of photoreceptors within the transduced area following subretinal delivery of Dual-AAV PEs.

The Dual-AAV Prime Editor platform effectively delivers Prime Editing components to photoreceptors with high efficiency, enabling the precise correction of pathogenic mutations that cause RP with no evidence of unintended edits. Updated results supporting the feasibility of the platform to address multiple gene targets in retinal diseases will be presented at the time of the meeting.

P0611

CRISPRa-mediated activation of HSC engraftment enhancers: towards the development of an epigenome-editing platform to control long-term engraftment of *ex vivo* genetically modified HSCs

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Autologous transplantation of genetically modified hematopoietic stem cells (HSCs) has emerged as a promising technology for the treatment of a variety of human blood disorders. *Ex vivo* HSCs genetic engineering relies on the stem cells isolation from the patient`s mobilized peripheral blood for the correction of the disease-causing mutation, followed by the re-infusion of corrected cells into the patient. A successful treatment requires the most primitive HSCs with long-term repopulating potential to home and engraft into the bone marrow niche, where they self-renew and establish a new population of genetically modified cells that pass on a correct copy of the gene to the progeny. While *ex vivo* HSC gene editing enables efficient genetic correction *in vitro*, it suffers from poor engraftment of corrected cells *in vivo*, thus limiting the therapeutic potential of HSC-based genetic therapies.

We hypothesise that the specific and transient activation of distinct HSC engraftment enhancer genes (EEGs) through epigenome editing could represent a promising strategy to reverse the perturbation of the HSC transcriptional program caused by *ex vivo* manipulation. To devise this CRISPR activation-based epigenome editing system (CRISPRa), we first selected potential EEGs among regulators of HSC migration and stemness maintenance and evaluated the impact of *ex vivo* manipulation on the expression of these genes. Primitive HSCs, multipotent progenitors and committed progenitors were FACS sorted and the expression level of EEGs was quantified before and after *ex-vivo* manipulation. We observed that the selected genes were highly expressed in primitive HSCs but lost during differentiation and throughout *ex vivo* culture. By exploiting the

ability of CRISPRa to induce a transient yet robust transcriptional activation of a target gene, we activated the expression of EEGs in HSCs by delivering a catalytically dead Cas9 fused to the tripartite regulator VPR (dCas9-VPR) as mRNA alongside two synthetic guide RNAs (sgRNAs) targeting the promoter region of the selected genes. Time-course analysis of EEGs mRNA and protein expression revealed a significant peak of gene activation 16h after CRISPRa delivery, which returned to baseline 96h later; this demonstrates that the platform is compatible with the kinetic of HSPCs homing and engraftment after transplant, while avoiding the potential negative effects of persistent gene expression perturbation in HSCs. Transient activation of EEGs led to increased homing and migration of epigenome edited HSCs in response to chemotactic cues and to expansion of more primitive HSCs in culture. Lastly, by devising an orthogonal gene editing - epigenome editing platform, we tested the efficiency of this system in providing a homing and engraftment advantage in vivo to HSCs gene-edited with a CRISPR/Cas9 platform for the treatment of Wiskott-Aldrich Syndrome.

Overall, our data demonstrated the feasibility of CRISPRa system for transient upregulation of genes involved in HSC engraftment. We now aim to integrate this platform to already established gene correction systems to boost gene edited HSC engraftment upon transplantation and to reach sensible gene correction rates *in vivo*, with the final aim of unlocking the full potential of HSC-based cell therapies for the treatment of blood disorders.

P0612

Journey to the Centre of the Airway: tackling cystic fibrosis with cell-penetrating VP22-ABEs

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Cystic Fibrosis (CF) is an autosomal recessive disorder caused by mutations in the *CFTR* gene, encoding for the CFTR anion channel protein expressed at the apical plasma membrane of epithelial cells that regulates transepithelial fluid movement. Due to the complex architecture of the lung pseudostratified epithelium (LPE), targeted lung gene therapy still faces major challenges.

Gene editing of the progenitor lung basal cells (BC) may have the potential to permanently correct any CF-causing mutation. Whilst luminal epithelial cells (LEC) can be readily transfected with DNA or RNA molecules encoding gene-editing constructs, the complex architecture of the LPE causes a potential impediment to the direct targeting of BC. Considering this, we decided to determine if gene-editing cargo delivered to the LEC could transfer the gene-editing ribonucleoprotein (RNP) complex to the nuclei of BC cells below.

Inspired by previous studies[1] using the HSV-1 VP22 cell-penetrating peptide, we developed VP22-GFP and VP22-adenine base editor (VP22-ABEs) fusion proteins. We hypothesise that VP22-ABE-RNPs may retain VP22's intrinsic nuclear localization and intercellular trafficking capacity, thus being capable of travelling through the LPE and successfully delivering gene-editing complexes to BC.

Expression vectors encoding for VP22-GFP, VP22-ABE8e(NG) and VP22-ABE9-SpRY, were designed using gBlocks encoding for a VP22 sequence cloned under control of a CMV promotor in the precursor expression vectors. To initially characterise pVP22-GFP, HEK293T cells were

transfected, and high levels of GFP expression were detected by flow cytometry 48h post-transfection. Moreover, a distinct nuclear localisation pattern of the protein was observed through fluorescence microscopy relative to the pGFP control that lacked VP22, thus confirming the nuclear localisation capacity of VP22-GFP fusion proteins. To assess the base editing profile of VP22-ABEs, we performed a characterisation step to assess the editing window of VP22-ABEs and precursor ABEs using 6 different gRNAs targeted to regions of the human genome present in HEK293T cells. A-to-G editing efficiency (%) was assessed 72h post-transfection by Sanger sequencing. ABE8e(NG) and VP22-ABE8e(NG) both displayed a 2-12 nucleotides (nts) editing window, with the highest editing levels achieved at adenine in position 5 (A5) of 77.7 and 64.4%, respectively. SpRY-ABE9 and VP22-SpRY-ABE9 both displayed a 4-6 nts editing window, with the highest editing levels achieved at A5 of 53.8 and 40.5%, respectively.

The current focus is now to determine the intercellular trafficking capacity of transfected VP22-ABEs in differentiated air-liquid interface (ALI) cultures of BCi-NS1.1 cells, which, due to their multipotent differentiation capacity, can be fully-differentiated under ALI culture conditions into different cell types of the LPE. With this approach, we will assess if VP22-ABE-RNP complexes produced in LEC can shuttle to the nuclei of BC and functionally correct the W1282X CF-causing mutation. For this effect, 72h post-transfection ALI cultures will be detached and dissociated from the transwell inserts to allow for the sorting of BC ($NGFR^{+ve}$) using flow cytometry. With this approach, we will compare the editing levels of VP22-SpRY-ABE9 and SpRY-ABE9 between the entire pool of cells and BC cells to determine the shuttle capacity of VP22-ABE-RNP complexes into BC.

[1]Elliott & O'Hare, (1999) Gene Therapy PMID:10341888

P0613

Correction and integrity of duplex base editing for fetal hemoglobin induction in B-Hemoglobinopathies

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Beta-haemoglobinopathies are the most common monogenic diseases. Of these, β-thalassemia results from decreased (β^+) or absence (β^0) of β-globin chain production, causing hemolysis and ineffective erythropoiesis. Elevated γ-globin levels, the fetal hemoglobin (HbF, $\alpha_2\gamma_2$), confer major clinical benefits in β-thalassemic patients. Recent research demonstrates that targeting HbF modifiers such as *BCL11A* and *HBG* genes, enhances γ-globin expression. Genome editing tools show therapeutic promise such as base editors (BEs) which are safer and likely more efficient than traditional DSB-dependent CRISPR/Cas technology. However, safety concerns regarding chromosomal aberrations still remain a risk. The project aims to adopt BEs for simultaneously editing both *BCL11A* erythroid enhancer and *HBG* promoter for correspondingly increased clinical potential and investigate the potential of genomic alterations using CAST-Seq analysis.In this study, the HUDEP-2 cell-line and patient-derived $CD34^+$ cells were used. Cells were nucleofected with three different guide RNAs (gRNAs) for single or double target editing. Editing efficiency and functional studies at the DNA, RNA, and protein level were conducted. Lastly, CAST-Seq analysis was performed, for the assessment of chromosomal aberrations. A successful double base editing protocol was established resulting in high editing efficiency after targeting both *BCL11A* and *HBG* loci. Our study reveals that multiplex base editing of both *BCL11A* enhancer and *HBG* promoter

(2xBE) in patient-derived $CD34^+$ cells induces robust γ-globin and HbF induction reaching to 56.86% HbF increase, indicating a potential therapeutic benefit of 2xBE approach. Our study shows that single and, most importantly, double base editing offers a safe editing option, resulting in a low incidence of genomic alterations in these therapeutically relevant target loci. In this pioneering study we demonstrate the effectiveness of multiplex base editing targeting both, *BCL11A* and *HBG* loci. This approach induces potent fetal hemoglobin with negligible chromosomal aberrations, highlighting the therapeutic potential and safety benefits.

P0614

Improving CRISPR base editing efficiency prediction through data generation and deep learning

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CRISPR base editing (BE) allows inducing point mutations, holding the potential to avoid the DNA repair challenges in gene editing. However, the main concern by applying base editors is the unwanted bystander edits, where multiple target nucleotides are likely to be edited simultaneously. Accurate prediction of bystander edits when designing guide RNAs (gRNAs) is crucial for BE gRNA design. To construct computational prediction methods, data of both high quality and in high number with high-efficient gRNAs are needed. However, these demands are not met with as the current datasets come with a high number of low-efficient gRNAs. To address the challenges, we first generated a pool of ~11,500 gRNAs for both adenine-BE (ABE, converting A•T into G•C base pairs) and cytosine-BE (CBE, converting C•G into T•A base pairs) using SURRO-seq technology. This increases the pool of available data to ~17,000 gRNAs for both ABE and CBE while low efficient gRNAs with less than 100 edited reads have been reduced. With the available data we construct a design tool which simultaneously predicts gRNA efficiency and outcome frequency. As the different data sets holds different densities from low to high efficiencies while overlapping in few specific gRNAs, we did not attempt homogenize them into a single coherent set, but rather devised a scheme that trains on three different available data sets simultaneously. Hence each data set comes with its own input (target DNA with context, edited outcome), output (efficiency and outcome frequency), and label for the given data set. Using this training strategy leads not only to enhanced performances on the individual data sets, while allowing users to make tailored prediction for the dataset most resembling one of the three used during training. In evaluating the performance we simultaneously evaluate the two-dimensional output of gRNA efficiency and outcome frequency by using a *K*-dimensional (K=2) versions Pearson and Spearman correlation coefficients. For training and testing the models we carefully ensured that there was no overlap between training and test sets and when comparing to other methods we ensured that they were evaluated only on gRNAs none of the methods were trained on. We evaluated a range of models with different data set labelling while comparing to published models and found that our BE efficiency prediction models CRISPRon-ABE/CBE outperform the existing ones. This can be exemplified by evaluation on the published Song test set where CRISPRon-ABE obtain Pearson correlation coefficient R_K (K=2) of 0.81 and the published DeepABE predictor obtain a value of 0.72.

P0615

CRISPR/Cas9-based precision B cell gene engineering produces active tissue nonspecific alkaline phosphatase for the potential treatment of hypophosphatasia

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Hypophosphatasia (HPP) is an inherited disorder characterized by defective bone mineralization, caused by deficiency of tissue nonspecific alkaline phosphatase (ALP). ALP is a dimeric zinc-containing metalloenzyme which hydrolyses inorganic pyrophosphate (PPi) and a variety of organic phosphates. Lack of functional ALP leads to elevated levels of PPi, which in turn inhibits bone mineralization, leading to osteomalacia/rickets (soft bones) and can be lethal without treatment. Currently, the only treatment for HPP is enzyme replacement therapy (ERT), asfotase alfa, requiring subcutaneous injections 3 to 6 times per week which is accompanied by injection site reactions including lipodystrophy. Terminally differentiated human plasma cells derived from genetically engineered B cells (B Cell Medicines, BCMs), offer decade long natural longevity, capacity for high levels of protein secretion, ability to engraft without preconditioning, and are re-dosable, making them an attractive platform to provide durable therapeutic proteins in adults, as well as children. We sought to harness the power of BCMs by engineering B cells to produce active ALP protein for HPP. In this study, primary human B cells were expanded, precision engineered by CRISPR/Cas9 genome editing with AAV-mediated homology directed repair (HDR) to insert an ALP gene expression cassette into various loci, including *CCR5* (a safe harbor locus) and *JCHAIN* (a gene highly expressed in plasma cells). Guided by an artificial intelligence (AI)-based protein structure design engine (i.e., AlphaFold2, an optimized version developed for dimeric proteins), ALP protein constructs were optimized for activity and stability by fusing ALP with different Fc fragments (ALP-Fc) coupled via a variety of linkers. A pharmacokinetic study in the mouse model showed *in vivo* stability of AI designed ALP proteins comparable to the current approved ERT, asfotase alfa. *Ex-vivo* engineered BCMs secreted active ALP proteins up to 200 ng/1e6 cells/24hr as measured by Legendplex assay. BCM produced ALP (ALP-BCM) has comparable specific activity to asfotase alfa and can abolish PPi induced mineralization inhibition in an *in vitro* osteoblast cell-based model. Finally, NOG-hIL6 mice treated with ALP-BCM exhibited active ALP as revealed via PPi substrate reduction following *in vivo* challenge. In summary, we demonstrated successful production of active ALP from our novel BCM platform. The potential therapeutic application of this unique biologic delivery system could afford a new treatment modality for HPP.

P0617

Methods for genome-wide detection of single strand breaks induced by gene editors reveals the specificity of SpCas9 nuclease domains and provides comprehensive lists of potential off-targets for Prime Editors

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Off-target assessment of gene editing is critical for evaluating the precision and specificity of the technology as it moves into the clinic. While methods to detect off-targets for SpCas9 and nuclease-based editors are more mature, detection and quantification of off-target risk of second generation nickase editors, including Prime Editors, are works in progress. To fill this need, we have developed the genome-wide single strand break detection method 3-Prime End liGation sequencing (PEG-seq) that can detect the single strand breakome of a gene editor. This method allows high sensitivity analysis of the differential nicking activity of the RuvC and HNH domains of SpCas9 and identifies the partially overlapping specificities of these nuclease domains. Using PEG-seq, we have analyzed the single strand break profile of more than 100 spacer sequences and used statistical models to identify the specificity determinants of the RuvC domain of the Prime Editor. These results point the way toward developing more robust methods for in silico risk assessment of editing reagents. Further, PEG-seq and related in cellulo methods allow for comprehensive characterization of potential off-target risks for Prime Editing strategies for clinical programs. Analysis of clinical and non-clinical programs with these methods underscores the specificity of Prime Editing and the rarity of off-target events for Prime Editing strategies.

P0618

EDSpliCE: AAV-deliverable enhanced-deletion RNA-guided nucleases applied to rescue a common *USH2A*-related splicing defect

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Retinitis pigmentosa is one of the leading forms of inherited retinal dystrophies, causing progressive vision loss in over 1.5 million individuals globally. Amongst the most frequent underlying genetic bases for autosomal recessive isolated and syndromic forms of this condition are pathogenic variants in the *USH2A* gene. In up to 4% of patients suffering from Usher syndrome, which is Retinitis pigmentosa and hearing loss, a recurrent deep intronic variant (*USH2A* c.7595-2144A>G) is found. This variant disrupts canonical mRNA splicing, resulting in aberrant or truncated protein.

Aiming to develop a safe therapeutic strategy for splicing modulation, we have developed the Enhanced Deletion Splicing Correction Editing (EDSpliCE) platform and applied it to target the described *USH2A* variant. This editing system is based on novel engineered enhanced-deletion synthetic RNA-guided nucleases (EDsRGNs). These small chimeric proteins, composed of the synthetic endonuclease sRGN3.1 and the human exonuclease TREX2, generate larger deletions at the target sites whilst using only a single gRNA. While ensuring meaningful perturbation of the disease-related DNA sequence, the nuclease engineering aims to incorporate important safety features.

To evaluate the efficacy of this approach, two newly engineered EDsRGN nucleases coupled with five different guide RNAs were tested for their splicing rescue potential utilizing minigene splicing assays. Therefore, HEK293T cells were co-transfected with an *USH2A* c.7595-2144A>G minigene and an EDsRGN-gRNA-encoding plasmid, followed by analysis of splice products. Thereby, up to 90% of correct *USH2A* transcript was detected after transfecting either nuclease and a gRNA directly targeting the variant.

These rescue experiments were additionally performed by delivering the nuclease-gRNA combination by recombinant adeno-associated viruses (rAAVs). The efficacy of AAV-mediated delivery was evaluated by on-target editing analysis after transduction of homozygous patient-derived cells. Effective delivery of the editing machinery via rAAV transduction could be achieved in different cell types and led to substantial splicing rescue.

Occurrence of adverse chromosomal translocations upon gene editing treatment was examined by interchromosomal junction PCR. This method to detect hybrid chromosomes showed nearly undetectable occurrence of chromosomal translocations with the EDsRGN variants as opposed to sRGN3.1, while next generation sequencing (NGS) proved comparable on-target editing rates. NGS-derived genomic deletion profiles moreover demonstrated the induction of enhanced and directional deletions by EDsRGN variants.

Collectively, the AAV-packageable EDSpliCE platform represents a promising, both effective and safe approach for therapeutic splicing modulation. Ongoing experiments include unbiased assessment of potential off-target effects, analysis of large-scale deletions and protein-rescue experiments in AAV-transduced retinal organoids harbouring a 3xFLAG-epitope tag fused to the C-terminus of endogenous *USH2A*, aiding protein detection.

P0619

A mutation-independent approach to treat autosomal dominant retinitis pigmentosa exploiting compact CRISPR effectors from the human microbiome

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Autosomal dominant Retinitis Pigmentosa (adRP) is one of the most common forms of inherited retinal dystrophy (1 in 4000) and is caused by heterogenous mutations in the *RHO* gene (>200 described mutations), encoding the rhodopsin photopigment. This high genetic diversity makes personalized editing approaches largely unpracticable and creates the need for an inclusive treatment alternative.

CRISPR-based programmable and precise editing tools transformed the gene therapy field, resulting in an increasing number of clinical trials and the recent approval of the first gene edited therapeutic product. Despite these achievements, the CRISPR-Cas toolbox currently available still presents important limits regarding safety, deliverability and genomic targeting coverage which hamper its full potential for *in-vivo* human therapeutic applications. To overcome these limitations, we exploited our proprietary discovery pipeline and sourced novel CRISPR systems from human microbiome reservoirs. Nucleases of various sizes were selected by using our *in-silico* PAM prediction tool and tested for their editing efficiency, creating a collection of highly active enzymes that broaden the targetable sites in the human genome, are characterized by improved safety and deliverability and can be tailored for specific targeting needs. Directed evolution platforms are further implemented to enhance targeting specificity such as allele discrimination.

We thus developed a mutation-independent strategy which leverages on the allele-specific knockout of mutated rhodopsin by simultaneously targeting a high-frequency non-pathogenic single nucleotide polymorphism (SNP) found in the first exon of RHO, to allow allele discrimination, and an intronic region of the gene (bi-allelic cut). The selected candidate nucleases demonstrate improved editing over the SpCas9 benchmark and are characterized by the absence of detectable genome-wide off-targets.

Given its small size, our lead candidate was packed together with corresponding sgRNAs targeting RHO into an optimized all-in-one AAV-vector which was injected subretinally in a humanized mouse model bearing the human RHO P23H mutant allele, demonstrating high levels of the desired modification at the target locus.

Overall, our uniquely tailored approach for the identification of highly active and specific novel CRISPR nucleases potentially allows to access multiple new genetic targets and has been proven to generate easy to deliver and efficient CRISPR editors for *in-vivo* applications.

P0620

Improved prime editing by DNA repair modulation and pegRNA engineering

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Prime editing (PE) is a genome editing technology comprised of a Cas9 enzyme fused with a reverse transcriptase (RT), and a PE guide RNA (pegRNA). The pegRNA provides a template for the RT to introduce a diverse set of edits into precise regions of the genome. However, PE efficiency and purity can vary widely across different edits, genomic targets, and cell types. Additionally, PE can introduce unintended editing outcomes, such as non-templated insertions and deletions (InDels), off-target editing, as well as increased pegRNA scaffold integration by RT read-through of the template. These unintended editing outcomes create safety issues for clinical

use that would reduce PE therapeutic application. To resolve these issues, we present two novel strategies that significantly reduce unintended editing outcomes of diverse PE systems. First, we demonstrated that combining small molecule inhibitors for both the non-homologous end joining (DNAPKi) and microhomology-mediated end joining pathways (PolQi) shifted the editing outcomes towards homology-driven repair which increased the precision of PE3, PE5, TwinPE and PEn by up to 9.8-fold. We observed by deep sequencing that combination of DNAPKi and PolQi reduced indels and imprecise PE frequencies while enhancing precise PE events in different cell lines and at almost all target sites and edits tested. Importantly, for PEn, the use of inhibitors also reduced the frequency of unintended off-target editing by 90%. Secondly, we demonstrated that introducing chemical modifications into pegRNA, such as abasic spacers and modified bases, reduced scaffold-integration of the pegRNA into the genome by blocking RT activity. The use of engineered pegRNAs with PEn diminished scaffold integration and improved precise editing by up to 3.5-fold in multiple cell lines and across multiple loci. Importantly, we proved that engineered pegRNAs improved precise editing in primary human hepatocytes and in mouse embryos, in clinically relevant loci. All together, these strategies address crucial safety issues of PE, hence advancing its potential clinical applications.

P0621

Daiji: the first integrated method to analyze single and double-strand breaks in GUIDE-seq and Digenome-seq data for unbiased assessment of editing outcomes

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Genome editing technologies offer the potential to develop genomic medicines for correcting monogenic disorders and restoring functional cellular activity. Our RNA Gene Writers utilize the mechanism of target-primed reverse transcription and have demonstrated increasing efficacy in both in vitro and in vivo models for liver-directed therapies for diseases such as phenylketonuria (PKU), alpha-1 antitrypsin deficiency (AATD), and Wilson's disease.

Assessing the genotoxic potential of these technologies prior to their entering the clinic is crucial, requiring thorough evaluation of both on-target and off-target potential, and any unintended mutations. While various in vitro and in cellula methods, like GUIDE-seq and Digenome-seq, exist to quantify editing outcomes with specialized toolkits for either assay, there is a lack of integrated methods that provide a robust, common, scalable, and standardized approach for analyzing both assays simultaneously. Moreover, none of the existing state-of-the-art tools can infer single-strand breaks in addition to double-strand breaks, a critical limitation for all upcoming technologies aimed at overcoming the need for inducing double-strand break repair.

To address this need, we present Daiji, a novel computational method designed to analyze both GUIDE-seq and Digenome-seq datasets and process experimental designs with single or double-strand genomic breaks simultaneously. Daiji contains read-level analysis paired with spacer-matching logic to report putative off-target mutations. Daiji also introduces a new normalized cleavage score (NCS) based on on-target sequencing depth relative to a control that quantifies editing outcomes and enables comparisons across different samples and conditions without the biases of confounding factors. We tested Daiji using simulated reads designed to cover a wide

range of editing outcomes, varying sequencing quality (using ART and wgsim read simulators), and sequencing depths (from 1X to 100X) with multiple randomizations. We assessed the precision and recall of Daiji, obtaining an average of 0.92 and 0.97 respectively, outperforming digenome-toolkit2 (the state-of-the-art method for Digenome-Seq analysis) that reached 0.69 and 0.59 respectively using 20x genomic coverage and sequencing error comparable to Illumina HiSeq X alignment rates. We also benchmarked our NCS, showing its superior accuracy (r2 = 0.97) against standard cleavage score (r2 = 0.48) as measured by correlation with the rank order of synthetic sites. Moreover, with Daiji and the simulation framework, users can quantify the number of replicates required to detect editing.

Overall, our integrated method provides an advancement in the standardization and scalability of genomic editing analysis, with superior precision and recall compared to other available methods, introducing a better quantification score to measure the cleavage score, and supporting power analysis for sample replicates. Daiji is a powerful tool to quantify the precision and safety of genome editing technologies for clinical and pre-clinical applications.

P0622

Spacer-nick gene targeting approach repairs disease-causing mutations with increased safety

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The CRISPR/Cas9 system is a powerful tool for gene repair and holds great potential for gene therapy to cure monogenic diseases. Despite intensive improvement, the safety of this system remains a major clinical concern. In contrast to Cas9 nuclease, Cas9 nickases with a pair of short-distance (38-68 bp) PAM-out sgRNAs preserve gene repair efficiency, while strongly reducing off-target effects. However, this approach still leads to efficient unwanted NHEJ-mediated on-target mutations that may cause tumorigenesis or abnormal hematopoiesis. Recently, we established a precise and safe 'spacer-nick' gene repair approach that combines Cas9^{D10A} nickase with a pair of PAM-out sgRNAs at a distance of 200-350 bp. In combination with adeno-associated virus (AAV) serotype 6 to deliver DNA repair templates, this approach leads to efficient gene correction in human hematopoietic stem and progenitor cells (HSPCs including long-term HSCs) and T cells with minimal unwanted NHEJ-mediated on-target mutations. Using the spacer-nick, we developed an approach to repair disease-causing mutations in the *HBB*, *ELANE*, *IL7R*, and *PRF1* genes. We achieved 20-50% gene correction efficiencies with minimal unwanted on-target mutations. Based on the in-depth off-target assessment, frequent unintended genetic alterations induced by classical CRISPR/Cas9 were significantly reduced or absent in the HSPCs treated with spacer-nick. Thus, the spacer-nick gene repair approach improves safety and suitability for gene therapy.

P0623

Optimization of the modular Pin-point™ base editing platform for an engineered Type V CRISPR-Cas effector

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The recent appreciation of the variable function of the natural diversity of CRISPR-associated nucleases has led to an interest in expanding the gene editing toolbox. The type V CRISPR-Cas system is particularly attractive for clinical development due to its smaller size than SpCas9 and its unique PAM specificities, permitting a greater variety of delivery vehicles and allowing for enhanced accessibility of editing sites not always attainable with type II CRISPR-Cas enzymes, such as Cas9.

Revvity's modular Pin-point™ base editing platform employs the delivery of either a nickase or deactivated Cas enzyme, a deaminase fused to an aptamer binding protein, and an aptameric guide RNA (gRNA) that assemble *in vivo* and act in conjunction to facilitate precise single nucleotide conversions. While originally developed using Cas9 nickase, the modularity inherent to the Pin-point platform permits swapping of components to achieve optimal editing results. Here, we demonstrate robust site-specific editing at different target loci using a deactivated CasONYX (dCasONYX) version of the Pin-point base editing platform. dCasONYX is an engineered version of dCasMINI developed by Epic Bio. It has an improved off-target profile compared to SpCas9, exhibits very low immunogenicity compared to Cas9 and is less than 1.5 Kb in size. Further utilizing the modularity of the Pin-point base editing platform, we show that additional components of the system such as different deaminases and aptameric gRNA scaffold configurations can be leveraged in combination with alternative Cas enzymes to adjust the base editing window; this is of particular advantage for applications including SNP correction and gene knockout at sites that are not usually accessible with the nCas9 configuration. Finally, we have significantly improved the design and optimized the delivery parameters of the dCasONYX version of the Pin-point platform through generation of IVT mRNA and achieving robust chemical synthesis of gRNA over 150nt in length, making this configuration suitable for application to therapeutically relevant cell types such as T cells and induced pluripotent stem cells (iPSCs).

P0624

Multiplexed CRISPR/Cas-based transcriptional modulation and gene editing using multiple CRISPR/Cas orthologues

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Targeted gene-specific transcriptional modulation can be achieved using CRISPR/Cas-based activation (CRISPRa) and interference (CRISPRi) by recruiting or directly fusing transcriptional regulators to nuclease-deficient Cas9 (dCas9). These techniques allow for completely transient, programmable transcriptional modulation and have previously been utilized to regulate cell states

and guide differentiation trajectories. In this study, we explore the expanding field of transcriptional engineering and RNA delivery by evaluating different combinations of dCas9 and transcriptional modulators. Specifically, we use dCas9 from *Staphylococcus aureus* and *Streptococcus pyogenes* to achieve orthogonal multiplexed transcriptional modulation on a complete RNA platform, where transcription of two target genes is upregulated while two are repressed. Furthermore, we investigate the combination of targeted nuclease-based knockout through ribonucleoprotein (RNP) delivery of AsCas12a with orthogonal transcriptional repression and activation. We also simplify this approach by implementing SpCas9 RNP-based knockout using truncated sgRNAs (tsgRNAs) for CRISPRa that do not support DNA cleavage. Our results indicate that the inclusion of a third Cas ortholog, Cas12a, for targeted knockout can be seamlessly combined with orthogonal gene regulation using dSpCas9 and dSaCas9. However, the use of SpCas9 for knockout and dSpCas9 for CRISPRa using tsgRNAs for CRISPRa was highly affected on the ratio of SpCas9 protein to sgRNA and a balanced ratio is crucial for balancing these two functions. Moreover, we find that the duration of *CD123* activation is shortened when using tsgRNAs in comparison to full-length sgRNAs. These findings demonstrate the versatility and significant potential of using multiple CRISPR/Cas systems for complex genetic engineering in a straightforward, one-step process that achieves both transient transcriptome modulation and permanent DNA modifications. The versatility of the orthologous dCas9-effectors could be used to circumnavigate targets with sparce protospacer adjacent motif (PAM) sites, allowing for a greater combination of target genes for orthogonal transcriptional modulation. We believe such sophisticated engineering techniques can be applied in regenerative medicine and cell therapies to enable more advanced treatments.

P0625

A single-step process for engineering hypoimmunogenic pluripotent stem cells with the Pin-point™ base editing platform

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Pluripotent stem cells (PSCs) hold great promise for the manufacturing of numerous advanced cell therapies. Off-the-shelf allogeneic products derived from PSCs engineered to be compatible with large cohorts of patients have the potential to dramatically broaden access to these therapies, however their sensitivity to DNA damage presents challenges for efficiently performing the complex genome editing operations necessary to realise much of their potential. Base editors represent a potential solution to these challenges due to their reduced genotoxicity compared to nuclease-based technologies. We have developed the Pin-point platform, which enables the modular assembly of base editors composed of DNA binding Cas and DNA modifying deaminase components associated via an aptamer encoded in the sequence-targeting guide RNA (gRNA). Owing to the aptamer-dependent recruitment of the deaminase component to target DNA sequences, the Pin-point platform uniquely allows multi-purposing of a single Cas nickase component for simultaneous multiplexed base editing and targeted transgene knock-in. Transient delivery of mRNAs encoding a Pin-point base editor composed of Rat APOBEC1 and SpCas9 nickase in combination with synthetic aptamer-encoding gRNAs achieved durable target protein knockout, and substantially improved cell viability, editing efficiency, and genome integrity following multiplexed base editing compared to CRISPR-Cas9 with no adverse impacts on pluripotency. To demonstrate the utility of the Pin-point platform for the engineering of

allogeneic PSCs we generated a panel of clonal hypoimmunogenic iPSC lines with a range of genotypes using an automated clone tracking and picking workflow. Hypoimmunogenic iPSC lines generated via both multiplexed base editing and simultaneous base editing with targeted transgene integration retained pluripotency and exhibited the expected reduced immune responses associated with their modified human leukocyte antigen (HLA) phenotypes when differentiated to therapeutic cell products. The Pin-point platform therefore represents a safe and efficient solution to simultaneously perform multiple genome engineering operations via a novel single step process compatible with downstream automation, offering the opportunity to dramatically streamline the development of allogeneic iPSC-derived cell therapies.

P0626

Insertion of a potent activator TAL1:GATA1 composite motif in the gamma-globin promoters using the prime editing nuclease system as therapeutic strategy for beta-hemoglobinopathies

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β-hemoglobinopathies (β-thalassemia and Sickle Cell Disease, SCD) are genetic disorders caused by mutations that affect the production of the adult hemoglobin β-chain. The disease severity is alleviated by the co-inheritance of mutations in the fetal γ-globin (*HBG)* promoters that generate activator binding sites (BSs) or disrupt repressor BSs, leading to elevated fetal γ-globin in adulthood.

Here, we exploit a prime editing approach based on the use of a SpCas9 nuclease-prime editor (PEn) allowing the disruption of repressors BSs in the - 200 and -115 regions of the *HBG* promoters by inserting a potent 18-bp-long TAL1:GATA1 motif. This composite motif is recognized by the potent TAL1 and GATA1 transcriptional activators and is frequently present in highly expressed erythroid genes.

First, we designed multiple pegRNAs in the -200 or in the -115 region of the *HBG* promoters and we selected the best performing ones that efficiently insert the TAL1:GATA1 motif in K562 cells. Importantly, we showed that PEn was more efficient than the original prime editing system using a Cas9 nickase (PE) by inserting the composite motif in the *HBG* promoters at higher frequencies. Besides precise insertion of the TAL1:GATA1 motif, we also observed imprecise edits that correspond mainly to the deletion of the repressor BSs coupled with the introduction of a complete or partial TAL1:GATA1 motif with other substitutions, insertions and/or deletions (InDels). Of note, these imprecise edits are still productive in terms of γ-globin reactivation. Moreover, the modulation of the cellular repair pathways using inhibitors of the non-homologous end joining (NHEJ) and/or the alternative-end joining (alt-EJ) repair pathways, allowed to reduce nuclease-mediated InDels and improve editing precision, enabling the introduction of the precise TAL1:GATA1 motif in of 40% of the *HBG* promoters. Due to the use of a PE nuclease, these strategies led to the formation of the simultaneous cleavage of the two identical *HBG* promoters with the deletion of the 4.9-kb intervening genomic region at a high frequency (~60%). The analysis of the editing profile of the hybrid *HBG* promoter generated after the 4.9-kb deletion

showed that, in the presence of inhibitors, 60 to 70% of the hybrid promoters contained precise edits, thus likely inducing γ-globin reactivation. Then, we developed clinically relevant RNA or ribo-nucleoprotein delivery methods of the prime editing components to achieve high editing efficiency. We achieved up to 70% of insertion of the precise TAL1:GATA1 in the *HBG* promoters upon RNA delivery in K562 cells.

Finally, we tested our optimized prime editing system in hematopoietic stem and progenitor cells (HSPCs) from SCD patients. Optimization of different mRNA quantity and ratios led to 10% precise prime editing and 7% of Indels when targeting the -200 region of the *HBG* promoters. Modulation of the different cellular repair pathways and further optimizations of the culture and transfection conditions in HSPCs are currently being tested to improve the purity of the prime editing products and further increase the editing efficiency in HSPCs with the goal of providing sufficient proof of efficacy for the treatment of β-hemoglobinopathies.

P0627

Personalized prime-editing therapies for metabolic diseases

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Although clinically diverse, metabolic diseases have the same molecular basis: genetic mutations. Advances in gene technology now allow genetic correction of many of these mutations. But how do we efficiently design therapies for all those individually rare diseases? We use a platform approach for personalized prime-editing therapies. Prime editing is a precise CRISPR-Cas9 based genome-editing technique creating single stranded DNA breaks. It consists of a prime editor nickase, guided to the DNA-locus by a prime editing guide RNA (pegRNA). To develop patient-specific therapies, we personalize the prime editor, pegRNA and delivery vehicle. To this end, we developed a fluorescent reporter system (fluoPEER) to screen pegRNAs, challenge their secondary structure, screen for silent mutations, relevant nicking gRNAs and optimize the prime editor to target that specific locus. We then personalize the delivery method. Depending on the target organ, we either use mRNA encapsulated in lipid nanoparticles (LNPs) or virus-like particles (VLPs). We select and optimize the best-performing therapy per mutation and target organ and even use a green to blue fluorescent (GFP>BFP) prime-editing reporter system to test functional delivery. We finally personalize the optimal strategy to the patient- and organ-specific context in patient-derived organoids of the target organ. Using our method, we successfully prime edited mutations causing multiple metabolic diseases (e.g. Crigler Najjar, Methylmalonic Acidemia, Mitochondrial disease) in patient-derived cells. We achieved LNP/modRNA editing in human liver organoids and eVLP/RNP editing of up to 60%-100% (for GFP>BFP) *in vitro* and are working towards *in vivo* experiments. By demonstrating efficient prime editing of multiple clinically relevant mutations using tailored prime editing constructs and delivery vectors, we show that it is PRIME TIME to enable transformative gene-correction therapies for patients with metabolic diseases!

P0628

Highly efficient base editing of human hematopoietic stem and progenitor cells with the Pin-point™ platform

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Hematopoietic stem and progenitor cells (HSPCs) are a foundational cell type for the development of engineered therapies. Given their susceptibility to DNA damage, it is crucial to employ gene editing technologies that minimize genotoxicity. Base editors offer an efficient strategy to mitigate the challenges posed by nuclease-induced double-strand breaks (DSBs), such as activation of DNA damage response and chromosomal aberrations. We have developed the Pin-point™ platform, which enables the modular assembly of the base editor, including a DNA binding Cas and a DNA deaminase, via the interaction between an aptamer binding protein fused to the deaminase and an RNA aptamer located in the sequence-targeting guide RNA (gRNA). Allowing modifications of the DNA without relying on the introduction of DSBs, the Pin-point platform enables complex genetic modifications in a single intervention as demonstrated in primary human T cells and iPSCs, where we achieved efficient base editing at multiple sites and simultaneous targeted transgene knock-in without compromising genome integrity. The advanced safety profile of this technology makes it well suited to HSPCs editing. By optimising reagent design and delivery conditions of a Pin-point base editor composed of Rat APOBEC1 and SpCas9 nickase mRNAs, we achieve up to 80% C to T conversion at the B2M locus with high levels of editing purity and very low incidence of indels, an indirect measure of DSBs occurrence. Using the optimised conditions, we then targeted two separate loci known to reactivate γ-globin expression: the erythroid enhancer of the repressor BCL11A and the BCL11A binding site in the HBG promoter. We achieved a high level of base editing at both loci that corresponded with an increase in γ-globin mRNA and protein expression. Edited HSPCs retained viability, immunophenotype, and differentiation potential toward the erythroid lineage *in vitro*. The ability to base edit HSPCs efficiently and safely, while retaining high cell viability and differentiation capability, demonstrates the strength of the Pin-point™ platform as a tool for the generation of advanced cell therapies using sensitive cell types.

P0629

Correction of All Exon 12 Variants Using a Cas12a Template-Jumping Prime Editing Strategy

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Exon 12 of *CFTR* hosts a range of different classes of cystic fibrosis-causing mutations. While exon 12 is one of the shortest exons (1.5% of the *CFTR* mRNA), it harbours 7.1% of all *CFTR* mutations (CFTR2). G542X alone contributes to 2.5% of all CFTR mutations, however the remaining exon 12 mutations occur with much lower frequencies. Due to the low frequency of each of these mutations, correcting all exon 12 mutations with a single gene editing strategy is an attractive approach. Recently, a prime editing variation called template-jumping prime editing (TJ-PE) was developed with the potential to replace longer sequences, typically 100-1000 bp, of a target genomic locus (Zheng et al., 2023). In this system, a TJ-PE guide RNA (TJ-pegRNA) directs

a prime editor to the desired protospacer sequence located upstream of the first mutation in target region, creating a single-stranded nick in the genomic DNA. This generates a free 3'-flap which anneals to the primer binding sequence (PBS) of the TJ-pegRNA, followed by reverse transcription of the reverse transcriptase template (RTT). This nascent DNA sequence is integrated into the genome aided by a downstream second PBS which anneals to a downstream 3'-flap generated by a nicking guide RNA on the opposite strand. A TJ-pegRNA with an RTT encoding exon 12 and a downstream sequence complementary to intron 12 should replace exon 12, therefore correcting all exon 12 mutations with a single pegRNA/nicking gRNA combination.

Due to the low GC-content of the intronic sequences proximal to *CFTR* exon 12, protospacer adjacent motif (PAM) sequences for traditional prime editors are infrequent. For this reason, we implemented AsCas12a, which recognises T-rich PAMs (Liang et al., 2024). In addition, the prime editing template (petRNA) portion of the TJ-pegRNA is split from the spacer sequence and circularised by ribozyme sequences, thereby limiting the intrinsic RNase activity of AsCas12a on the RTT. Finally, the RT is fused to an M-coat protein (MCP-RT) which recognises and tethers to an MS2 aptamer sequence on the circular TJ-petRNA (Liu et al., 2022). These adaptions reduce the proximity between cut sites in intron 11 and 12, as well as the length of the exon 12 replacement RTT. Implementation of this strategy results in whole exon 12 replacement in a low percentage of amplicon reads, which is improved with the simultaneous treatment with a DNA-PK inhibitor which limits non-homologous end joining. Editing efficiency across the replacement locus is even, suggesting no decay of editing efficiency across the RTT. Furthermore. no indels or substitutions were detected outside of the cut sites, suggesting that editing is confined to the replacement sequence. Future work endeavors to improve the efficiency of this strategy.

P0630

Development of a base editing mediated knock-in (BEKI) system for the non-viral generation of multiplex gene edited CAR T cells

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The CRISPR-Cas system enables precise genetic modifications, including the insertion of DNA transgenes at specific genomic sites through homology-directed repair (HDR). Gene editing has been utilized to redirect T cells towards tumor cells by inserting a chimeric antigen receptor (CAR) into the *T cell receptor alpha constant* (*TRAC*) gene, resulting in favorable functionality due to physiological regulation of the CAR. Additionally, enhancing the ability of CAR T cells to resist exhaustion and immunosuppressive microenvironments may require further/additional genetic modifications. We and others have demonstrated how multiplexed gene editing can enable cells to evade allo-immunity in immunocompetent hosts, paving the way for efficient off-the-shelf cell therapies. Yet, the development of complex genomic interventions increases the risk of genotoxic events due to off-target editing and on-target genomic aberrations. Utilizing Cas9 nickase (nCas9) mediated HDR has been proposed to reduce undesired off- and on-target editing compared to conventional gene editing methods relying on the introduction of DNA double strand breaks (DSB). However, nCas9 is ineffective for simultaneous knock-in (KI) and multiple knock-outs (KOs), resulting in a need for more efficient solutions. The fusion of deaminase enzymes to nCas9 can mediate DSB-free base editing of adenine and cytosine with nucleotide resolution.

Inactivating splice sites or introducing stop codons using base editors (BE) mediates highly efficient protein disruption, while limiting genotoxic events during multiplexed applications.

In this study, we propose a system for base editing mediated knock-in (BEKI) that utilizes the nCas9 domain of the BE to introduce paired DNA nicks on opposite strands to induce HDR mediated transgene insertion. Preliminary experiments using the BEKI system demonstrated the feasibility of precise CAR KI into the *TRAC* locus, while further optimization regarding guide RNA (gRNA) selection and corresponding nick positioning was required to improve the initial editing efficiency. We observed a drop in editing efficiencies when the distance between the introduced nicks exceeded 200 base pairs. Furthermore, higher frequencies of specific base changes within the editing window determined by the gRNAs correlated with reduced editing efficiency, as nCas9 binding diminishes with an increasing number of gRNA mismatches. Selecting gRNAs with limited editable bases significantly improved the KI efficiency. By screening gRNA pairs with adjusted HDR templates, we identified crucial BEKI design parameters and gRNA combinations for efficient CAR KI into *TRAC*. Due to its favorable safety profile and inherent base editing capabilities, the BEKI system is well-suited for introducing multiple genetic modifications simultaneously. Using BEKI, we generated multiplex gene-edited CAR T cells with enhanced properties. Functional evaluations of these CAR T cells, alongside ddPCR monitoring for chromosomal translocations between edited loci, confirmed the safety and efficacy of the multiplexed BEKI strategy.

The BEKI system enables the efficient and safe generation of multiplex gene-edited CAR T cells, enhancing their functionality and persistence for off-the-shelf use. Relying on a single gene editing tool potentially facilitates the GMP-grade manufacturing for clinical applications.

P0631

Targeted knock-in of *NCF1* cDNA into the *NCF2* locus leads to myeloid phenotypic correction of p47^{phox}-deficient chronic granulomatous disease

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p47^{phox}-deficient chronic granulomatous disease (p47-CGD) is a primary immunodeficiency caused by mutations in the neutrophil cytosolic factor 1 (*NCF1*) gene that encodes for the p47^{phox} subunit of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. Due to the resulting defective NADPH oxidase function in phagocytes, CGD patients suffer from life-threatening bacterial and fungal infections, as well as hyperinflammation. Here, we explore gene editing in hematopoietic stem and progenitor cells (HSPCs) to cure p47-CGD.

We used CRISPR-Cas9 to insert a construct consisting of a 2A oligopeptide and the *NCF1* cDNA (2A-*NCF1*) into the 3' end of the *NCF2* gene. *NCF2* encodes for p67^{phox}, a subunit of the NADPH oxidase that is highly expressed in myeloid cells. Upon homology-directed repair (HDR)-mediated knock-in of the *NCF1* cDNA into the *NCF2* locus, the expression of endogenous p67^{phox} will result in the production of separate transgenic p47^{phox} in the myeloid compartment by the action of 2A ribosomal skip. To target HSPCs, we performed electroporation of Cas9 ribonucleoprotein and delivery of the 2A-*NCF1* template by integration-deficient lentiviral vectors (IDLVs), or by adeno-associated viral vectors (AAVs).

Knock-in of the *NCF1* cDNA at the *NCF2* locus successfully corrected p47^{phox} deficiency, leading to the reconstitution of NADPH oxidase function in edited p47-CGD patient HSPCs and in p47^{phox}-deficient mouse HSPCs. Transgenic p47^{phox} expression under the control of *NCF2* followed a myeloid differentiation pattern. Upon knock-in in human HSPCs, the fraction of phenotypic long-term HSCs (CD34+ CD38- CD45RA- CD133+) was unchanged, and cells retained clonogenic potential upon *in vitro* secondary CFU replating. Endogenous p67^{phox} expression was not affected by the editing and no fusion proteins of p47^{phox}-2A-p67^{phox} were detected.

To assess safety of the knock-in treatment, we characterized the quality of vector integration events and CRISPR-mediated off-targets using a combination of droplet digital PCR copy number determination, linear-amplification mediated PCR, and CHANGE-seq. Apart from HDR-based knock-in, we detected unintended integrations containing viral-derived elements in the edited cells that could be mediated by non-homologous end joining (NHEJ)/ microhomology-mediated end joining (MMEJ). These integrations are likely due to residual integrase activity of IDLVs as the integration sites were found mostly in intronic regions and were enriched downstream of transcriptional start sites. In addition, off-target (OT) sites identified by CHANGE-Seq did not match the OT integration sites, suggesting that these unintended integration events were not mediated by Cas9 activity.

In conclusion, we present a gene therapy strategy for p47-CGD, linking the *NCF1* cDNA to the *NCF2* locus, ensuring spatiotemporal regulation of the transgene limited to myeloid cells. However, our findings on off-target integrations emphasize the need for comprehensive safety evaluation of post-editing outcomes, prior to clinical application of nuclease- or viral vector-based therapies.

P0632

Immunodeficient Mouse Models to Assess Human HSPC Engraftment and Gene Modification: NSG *vs*. NBSGW

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Humanized mouse models are essential in the assessment of long-term engraftment of human hematopoietic stem and progenitor cells (HSPC) and the persistence of genome modification. The NSG strain permits the engraftment of human HSPC after cytoreductive irradiation. The NBSGW mouse model is increasingly utilized for investigating the effects of genetic manipulation in human HSPC, particularly for hemoglobinopathies. Unlike the NSG mouse, the NBSGW model allows for high and sustained levels of human cell engraftment without conditioning, as well as the

production of human erythroid progenitor cells in the bone marrow (BM). These critical differences led us to investigate engraftment and gene modification outcomes from different gene therapy approaches (lentiviral vector [LVV] and gene correction) in both mouse models.

Mobilized peripheral blood CD34+ HSPCs were transduced with an LVV-GFP and subsequently sorted based on GFP expression (untransduced [GFP-Neg], GFP-Low, GFP-Mid, and GFP-High). Sorted cells were transplanted into NBSGW or busulfan-conditioned NSG mice at equivalent doses to evaluate the engraftment potential of the different cell populations across the two models. While the NSG model showed defects in the engraftment potential of highly transduced cell fractions ($p<0.05$), the NBSGW model demonstrated a modest though statistically significant decrease in engraftment between the GFP-Neg group (77% hCD45) and GFP-High group (64% hCD45, $p<0.05$); the GFP-Low and GFP-Mid group engraftment efficiencies were equivalent to the GFP-Neg group.

Mobilized peripheral blood CD34+ HSPC were edited using CRISPR/Cas9 to correct the *HBB* sickle mutation and transplanted into NBSGW or sub-myeloablative irradiated NSG mice at equivalent doses to assess engraftment capacity and gene correction outcomes. 16-weeks post-transplant statistically significant differences were observed in human engraftment between NSG (55%) and NBSGW (83%) mice ($p<0.05$), while gene correction at the BM by homologous directed repair (HDR) was comparable between the two strains. Edited CD34+ HSPC were transplanted into NBSGW or sub-myeloablative irradiated NSG mice at limiting doses showing a significantly greater SCID repopulating capacity in the NBSGW model; while gene correction and allelic disruption frequencies were comparable between the two mouse models at all cells doses.

Single-cell RNA-sequencing was performed on lineage depleted and CD34+ enriched human CD45+ cells isolated from murine BM of both mouse strains. Four distinct populations classified as CLP, CMP, GMP and MEP were observed only in NBSGW mice. The presence of these populations implies a greater capacity of the NBSGW model to support human hematopoiesis from the long-term engrafting HSC resulting in an overall larger proportion of human cells.

These data suggest that both the NBSGW and NSG models allow for assessment of gene modification in long-term engrafting HSC. However, the significantly higher repopulating capability of the NBSGW mouse may mask cell populations with engraftment deficits due to genotoxicity, as observed in the LVV-GFP transplants above. This does not appear to be the case with site-specific gene editing, in which modifications are theoretically occurring at a single locus in the genome. Proper selection of *in vivo* models for gene therapy are critical in the evaluation of cell products prior to clinical translation.

P0633

End-to-end Tools and Services for Interrogation of CRISPR-Cas Associated Genotoxicity

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Identifying and verifying low frequencies of genomic alterations resulting from off-target editing, gRNA synthesis errors, cross-contamination, or other unintended gRNA activity is critical to preventing unexpected genotoxic effects for the gene editing therapeutic community. However, assembling the necessary components and expertise for genotoxicity characterization studies is expensive and labor intensive. To better enable the gene editing community, we demonstrate a

series of tools, workflows, and services that can be leveraged to perform genotoxicity characterization of CRISPR reagents. First, we demonstrate an empirical *in vivo* tag-based off-target nomination service rooted in our RNaseH2-dependent amplification technology as a method to generate an off-target list for a target gRNA. Then, we demonstrate how outputs of this service can be fed into rhAmpSeq to identify and classify indels and chromosomal re-arrangements at on/off-target loci with increased effectivity compared to existing methods. Finally, we demonstrate a method to sensitively identify gRNA cross-contamination down to sub-0.1% gRNA contamination levels by leveraging unique NHEJ DNA repair fingerprint information of select gRNAs. Using this method, we demonstrate that editing attributable to a characterized gRNA activity can be identified at frequencies far below detection limits of canonically utilized tools, and we further use this method to characterize the ability of IDT's synthesis platform to prevent cross-contamination reproducibly. From this work, we demonstrate the importance of manufacturing processes in mitigating unintended genomic alterations and present new tooling/services to improve genotoxicity characterization in gene therapy workflows.

P0634

Multiple *COL7A1* gene exon excision as therapeutic strategy for RDEB using a CRISPR/Cas system

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Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a rare genetic disorder characterized by the absence of structural integrity of the skin. This results from an abnormal production of collagen VII (C7) protein, skin structural protein, that arise from the mutations in the *COL7A1* gene. Independent or multiple excision of the theoretically skippable exons can be achieved using a CRIPSR/Cas system. With this, excision of exons containing mutations that cause RDEB can be performed to obtain the edition of the cells and thus, a permanent restoration of functional C7 production. This study aims to target, among others, the most common mutation in Irish population (located in exon 31, also found in Australia, UK and the USA) and through multiple exon excision, encompassing a wider range of mutations within *COL7A1* gene. In this study, independent excision of exons 31 and 34 and multiple excision from exon 31 to 34 were performed, in immortalised N/TERT-1 human keratinocyte cell line, by delivering the CRISPR/Cas9 system, with previously designed guide RNAs (gRNAs), via electroporation. The editing efficiency resulted in more than 80% in independent excision for both exons and 60% for multiple excision (exon 31 to 34). Deletion of the exons was confirmed by Sanger sequencing. Subsequently, production of C7 in edited cells was semi-quantified through western blot and immunocytochemistry, confirming exon excision do not disrupt protein production. Furthermore, N/TERT-1 cells with an induced premature stop codon (PTC) mutation on exon 31 that leads to an absence of C7 production was generated to confirm 64% restoration of C7 protein after exon 31 excision when compared to wild type N/TERTs. Additionally, RT-qPCR was performed to analyse the exon deletion at a mRNA level. With this, exon 31 transcription was found absent in edited N/TERT-1 for independent exon 31 excision and considerably reduced for exon 34 independent excision when compared to untreated cells. Herewith, this study confirms efficient and feasible independent exon 31 and 34 excision and multiple exon 31 to 34 excision *in vitro* and production of C7 as a potential therapeutic strategy for the Irish EB population.

P0635

Neuron-selective promoters improve tolerability of AAV-vectored in vivo gene editing for latent HSV infection

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The peripheral nervous system (PNS) is an attractive target for gene therapy, potentially allowing improved management of chronic pain, neurodegeneration, inherited disorders, and certain infections. Adeno-associated virus (AAV) vectors can efficiently target the PNS and mediate robust transgene expression. However, AAV vectors provide limited selectivity for the PNS, leading to undesired transgene expression in other tissue types. Furthermore, AAV has the potential to cause hepatotoxicity, and a more recently-appreciated potential to trigger PNS neurotoxicity. Our laboratory has been using AAV-vectored meganucleases (MN) for the durable control of herpes simplex virus (HSV) infections, via direct gene editing of latent HSV genomes residing in somatic and autonomic ganglionic neurons of the PNS. We routinely observe a 1-2 log reduction in ganglionic latent HSV viral load in latently-infected mice after therapy, which translates into a similar reduction in HSV shedding upon viral reactivation in infected animals. However, we have observed delayed weight gain, hepatotoxicity, and PNS histopathologic changes at high AAV doses in this disease model. To limit therapy-associated hepatotoxicity, we preferentially targeted transgene expression to ganglionic neurons, and compared meganuclease expression in ganglionic vs. liver tissue after intravenous injection of AAV9 vectors using either the constitutive CBh promoter or a neuron-selective promoter from the calmodulin kinase II gene fused to the CMV enhancer (E/CamKII). Overall therapy efficacy and tolerability were also compared between the promoters. We found that treatment with AAV-CBh-MN led to robust meganuclease expression in ganglionic tissue as well as in liver. In contrast, AAV-E/CamKII-MN allowed strong meganuclease expression in ganglionic tissue only, with no detectable expression in liver. AAV-E/CamKII-MN therapy was better tolerated, with treated animals gaining weight normally, in contrast to delayed weight gain observed after administration of AAV-CBh -MN. In agreement with this finding, liver inflammation was observed after administration of AAV-CBh-MN, but absent after administration of AAV-E/CamKII-MN. Histological changes consistent with neurodegeneration were observed in somatic and autonomic ganglia after treatment with AAV-CBh, but again absent with AAV-E/CamKII-MN, raising the intriguing possibility that PNS toxicity after AAV therapy might result from transgene expression in non-neuronal cells, rather than a direct neurotoxic effect. On a per vector genome basis, both transgene expression and therapeutic efficacy against latent HSV were greater with AAV-CBh-MN, but this effect was outweighed by the improved tolerability of AAV-E/CamKII, allowing increased AAV dose. Taken together, these results support the use of tissue-selective promoters to limit transgene expression to desired tissues and improve gene therapy selectivity and tolerability.

P0636

Disease modeling and treatment of cystic fibrosis using 2i technology

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Cystic fibrosis (CF) is a genetic disorder resulting from a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, impacting the lungs, pancreas, and other bodily organs. Existing CF treatments such as Trikafta and gene therapy approaches target a specific patient subset, necessitating additional therapies or re-administration. Although genome editing with CRISPR-Cas9 has been utilized to correct the CFTR gene, its in vivo efficiency remains limited. AstraZeneca has developed a method for precise genomic insertions employing the SpCas9 nuclease-based prime editor, PEn, but more work is needed to optimize the system for therapeutic use. This approach combines PEn with canonical pegRNAs to induce homology-dependent double-strand break repair, leading to precise insertions (Peterka et al., 2022). Nonetheless, the generation of cell line models for CF is crucial for mutation correction. In this study, cell line models were created utilizing a colon cancer cell line for two prevalent cystic fibrosis mutations using a single guide RNA and a single strand donor template, thereby facilitating homology-directed repair (HDR). Furthermore, our laboratory introduced a new technology, "2i technology," demonstrating that concurrent inhibition of DNA-PK and PolΘ enhances precise genome editing (Wimberger et al., 2023). Cells were pre-treated with these inhibitors, leading to improved editing efficiency. Subsequently, we intend to optimize PEn approach to develop a therapeutic genome editing treatment for CF in human cell lines and patient-derived intestinal organoids. We also plan to employ AstraZeneca's proprietary lung-targeting lipid nanoparticles (LNP) to evaluate PEn *in vivo* in mice and conduct safety and toxicity assessments.

P0637

Prime editing with mRNA and lipid nanoparticles to repair inherited metabolic errors

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Prime editing is a promising CRISPR/Cas9-based gene-editing tool that mediates highly precise modifications of DNA without double-strand DNA breaks or donor DNA templates. The precision and versatility of prime editing hold great promise for gene-correction therapies for metabolic diseases. However, delivery of the prime-editing tools is challenging because their size exceeds the capacity of commonly used viral vectors.

We explored use of mRNA encapsulated in lipid nanoparticles (LNPs) to mediate transient expression of prime-editing tools in the liver, the main metabolic organ. We selected a point mutation causing *MMUT*-type methylmalonic acidemia (MMA), present in a well-characterized mouse model, to investigate *in vivo* translation of prime editing technology. To this end, we designed and tested different prime-editing guideRNAs (pegRNAs) and nicking guideRNAs (ngRNAs), produced mRNA of these and the prime editor via in vitro transcription and formulated LNPs through microfluidics. We tested gene-editing in different cell-models, using flow cytometry for our fluorescent reporter system (fluoPEER), as well as ddPCR and DTECT, to quantify genomic editing efficiency.

We were able to generate pegRNAs that allowed editing of the *MMUT*-mutation in a mouse cell line with 30% efficiency. We optimized mRNA production and formulation of LNPs for mRNA

delivery in rat hepatocytes, human hepatocytes, HEK293T cells, HepG2 cells, and mouse fibroblasts, resulting in an average of 30% editing efficiency *in vitro*.

In this proof-of-concept study, we demonstrate that we can generate mRNA of the prime-editing tools encapsulated in LNPs to gene-edit different (liver) cell lines with an efficiency that we anticipate to be clinically relevant. Upon validation in animal models, we expect that this strategy may be used to develop truly transformative therapies for patients with (liver dominant) metabolic diseases.

P0638

Leveraging precise genome editing through homology-independent targeted integration (HITI)

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Homology-independent targeted integration (HITI) is a form of genome editing that allows precise insertion of arbitrary sequences via the NHEJ pathway and can be used to disrupt and replace dominant gain-of-function mutations. The HITI approach was investigated as a potential therapeutic strategy for addressing pathogenic mutations in the human *SERPINA1* gene which encodes alpha-1 antitrypsin (AAT). Wild type AAT is produced in the liver and is secreted via the circulation to the lungs, where it functions as a serine protease inhibitor. AAT primarily inhibits neutrophil elastase in the lungs to regulate the inflammatory immune response. Misfolded aggregates of AAT, caused by pathogenic mutations, lead to liver damage through the (gain-of-function) generation of inclusion bodies and also lung damage through loss of wild-type anti-protease function. Here, we modelled therapeutic interventions at the *SERPINA1* locus by integrating a fluorescent reporter transgene (*mNeonGreen)* into the *SERPINA1* exon 1 at a position ideally suited to correct a range of pathogenic mutations. Using droplet digital PCR, a successful integration of the transgene was achieved with an efficiency of 6.3% in cells (HEK293T HITI reporter cell line). Upon investigation, a subset of edited cells showed insertions and or deletions (indels) at the Cas9/gRNA mediated double-strand break sites. However, Amplicon-EZ next generation sequencing (NGS) of 80,000 samples confirmed that these indels were most often limited to only 5–12 bp in length and did not interfere with the desired transgene editing outcome, because the indels exclusively fell in untranslated intronic sequences. Interestingly, a subset of sequences, also identified by NGS, were initially thought to harbour larger indels within part of the inserted *mNeonGreen* expression cassette. In contrast, high-resolution melting (HRM) analysis of such sequences found there was no significant difference in melting temperature (Tm) between such samples and Sanger sequence verified TOPO cloned sequences ($p>0.9999$, $n=9$). The precision of HRM analysis was demonstrated by comparing TOPO clones harbouring sequence-verified indels, with significant differences in Tm observed with as little as 5 bp difference in 331 bb of analysed sequence ($p<0.0001$, $n=9$). Thus, the HITI approach was able to alter the *SERPINA1* locus by inserting a 1.6 kb reporter transgene via NHEJ without indels affecting the function of the inserted sequence. Adapting this editing approach to use a therapeutic *SERPINA1* cDNA has the potential to address AAT deficiency. This approach potentially can be used to address other dominant gain-of-function disorders where disruption of pathological sequences and replacement with fully functional native sequences are simultaneous therapeutic goals.

P0639

Zip Editing: an easy-to-use tool to increase CRISPR-Cas9 HDR-editing efficiency

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Genome editing using CRISPR-Cas9 holds great promise in the treatment of genetic diseases as a safer alternative to additive gene therapy. Cas9 nuclease is very efficient to introduce double-strand breaks. Those are repaired by end-joining pathways resulting in insertions and deletions (InDels) or by homology-directed repair (HDR) using an exogenous template to introduce a desired modification. The latter repair mechanism is less frequently used by cells because it occurs only in S-G2 cell cycle phases and depends in part on the availability of the exogenous template at the site of editing at the moment of the repair. To increase the presence of the exogenous template delivered as single-strand DNA oligonucleotides (ssODN), several solutions has been developed to import it with the RNP complex (Cas9 + gRNA) but mostly rely on modifications of the Cas9 nuclease to link the ssODN. We propose a new editing tool, called Zip-Editing (ZE) to import the ssODN template with the RNP complex that doesn't rely on Cas9 modification. It is also not based on the modification of the cell cycle, or of DNA repair mechanisms, which can have deleterious genotoxic effects and are a major safety concern in the development of gene therapies. Thus, this system can be used with commercially available Cas9s, gRNAs and ssODNs and can also be very easily adaptable to a new target to introduce any type of modification. We tested ZE to edit precisely different targets (eGFP, UROS, CFTR) in several cell lines (HEK-293T, K562) and in primary cells (human foreskin fibroblasts, human pulmonary basal and nasal cells, human hematopoietic stem and progenitor cells) for some relevant for gene therapy of genetic diseases. We reached an increase in HDR-editing efficiency up to 12-fold as compared to a condition where ssODN template is not imported with the RNP complex. These results are very encouraging to impose ZE as a new tool to precisely edit the genome and to be part of the gene editing toolbox to treat or model diseases.

P0640

Large scale manufacturing of a gene editing-based protocol for Pyruvate Kinase Deficiency hematopoietic stem cell gene therapy

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Pyruvate kinase deficiency (PKD) is an autosomal recessive blood disorder caused by mutations in the *PKLR* gene. This gene encodes the erythroid pyruvate kinase (RPK) enzyme, which controls

the last step of glycolysis in erythrocytes. PKD-erythrocytes suffer from energy imbalance caused by the reduction of the RPK activity leading to their premature destruction. PKD is associated with hemolysis, reticulocytosis, splenomegaly, iron overload, and may be life-threatening in the most severe cases.

The use of allogeneic hematopoietic stem and progenitor cells (HSPCs) to treat genetic blood cell disorders is a potential curative treatment but limited by donor availability and complications. Therefore, we developed a knock-in gene editing strategy by combining CRISPR/Cas9 and adeno-associated viral vector (rAAV6) donor delivery into hematopoietic stem and progenitor cells (HPSCs) for autologous transplantation.

Improvements in gene the editing protocol increased the targeted integration frequency in HSPCs up to 60%. No significant alterations in colony-forming unit potential and maintenance of the clonal repertoire, defined with a therapeutic DNA barcode donor were observed. Furthermore, gene edited HSPCs engrafted into immunodeficient mice and a polyclonal repertoire within the human hematopoietic chimerism of the gene edited cells was identified *in vivo*.

Scale-up of the optimized gene editing protocol has been performed in mPB-HSPCs from four different healthy donors (HD) and one PKD patient. One HD and the PKD patient drug products were performed under good manufacturing practice (GMP) conditions in different laboratories (Europe and Asia). Drug products accomplished quality specifications, with >80% cell viability and ability to generate committed progenitors. Over forty percent knock-in efficacy was obtained among the different mPB-HSPC samples consistently.

To sum up, here we present an optimised and validated GMP hematopoietic stem cell gene editing protocol, clinically applicable, for the treatment of PKD patients. Discussions with regulatory agencies are ongoing to start a First-in-Human knock-in gene editing clinical trial to genetically correct PKD patients in a precise and efficient manner.

P0641

Cas9-containing virus-like particles facilitate the production of edited CAR-T cells using a one-step transduction approach suitable to cGMP

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In recent years, numerous clinical trials and preclinical studies have demonstrated the efficacy and promise of Chimeric Antigen Receptor (CAR) T-cell immunotherapies as a therapeutic approach for haematological malignancies and solid tumours. However, the same studies have highlighted biological and manufacturing shortcomings that can limit the expansion and persistence of CAR-T cells in vivo. Different approaches are being pursued to improve CAR-T cell therapy. In particular, novel tools such as CRISPR Cas9 gene editing and novel base editing approaches are compelling for modulating T-cell phenotypes by knocking out genes detrimental to CAR-T cell expansion and persistence or genes causing unwanted fratricide or non-specific killing. Currently, electroporation is the most commonly used method to deliver Cas9-sgRNA ribonucleoprotein (Cas9-RNP) complexes into cells. Although highly efficient, this process extends manufacturing time, requires specialised equipment, increases costs, and results in poor cell recovery post-electroporation, which may be problematic for some patients due to disease status or prior therapeutic

treatments. Thus, novel Cas9-RNP delivery methods are needed. To address this issue, we developed virus-like particles (VLP) packaging Cas9-RNP complexes by fusing a codon-optimized Cas9 sequence to the codon-optimized human immunodeficiency virus type 1 (HIV-1) gagpol open reading frame via a link peptide containing the HIV-1 matrix and capsid cleavage site. Cas9-containing VLP were produced using a three-plasmid system consisting of one plasmid expressing HIV gagpol-Cas9 and sgRNA, a second plasmid expressing HIV Rev, and a third plasmid expressing the baboon endogenous virus envelope glycoprotein (BaEVRless) protein. The sgRNA with a scaffold containing an extended tetraloop stem and without a premature termination sequence was modified to include a Hepatitis D virus ribozyme and an HIV-1 Rev-responsive element at the 3' end, allowing efficient nuclear export and transport to the cytosol of producing cells where association with gag-pol-Cas9 will likely occur. These Cas9-containing VLP were produced using our standard lentiviral vector production method, which uses a serum-free suspension cell line, SJ293TS-DPB, and purified and concentrated using a combination of anion exchange and tangential flow filtration. We evaluated Cas9-VLP against clinically relevant targets in simultaneous co-transduction of healthy human donor T cells with vesicular stomatitis virus G protein pseudotyped lentiviral (LV) or Gibbon ape leukaemia virus pseudotyped γ-retroviral vectors (γRV) encoding CAR. We achieved editing efficiency above 70%, maintaining VCN similar to CAR-vector alone. Comparing Cas9-VLP with electroporation, cell recovery after editing was significantly lower in electroporated cells than in Cas9-VLP treated cells. We demonstrated that simultaneous delivery of Cas9-RNP and CAR-expressing vectors reduces manufacturing timelines and can be used in cGMP processes as an alternative to electroporation.

P0642

Enhancing safety and transgene expression for effective paracrine cross-correction with HSPCs gene editing

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Lentivirus-based *ex-vivo* gene therapy for hematopoietic stem and progenitor cells (HSPCs) has advanced notably in enabling paracrine cross-correction strategies but concerns regarding insertional mutagenesis and genotoxicity remain. Our study aims to achieve high transgene expression levels in myeloid cells by precise genome targeting of HSPCs. We developed an optimized editing platform by selecting a poised safe harbour locus within a bivalent region on HSPCs, which provides a favourable epigenetic regulation of the transgene. We searched for a target locus with bivalent and poised chromatin marks during HSC stemness that become active after myeloid differentiation, and selected CX3CR1. Editing of cord blood HSPCs was optimised to achieve efficient and specific targeted integration of our reporter (SFFVp-GFP cassette). We

delivered CRISPR-Cas9 as ribonucleoparticle, and tested different AAV6 donor templates, to exploit diverse pathways of integration. To demonstrate that our target-integrated cassette was downregulated during the early HSPCs stages of differentiation, we examined the GFP expression across different HSPC subpopulations, with especial focus on undifferentiated HSPCs and committed myeloid cells. Finally, we assessed the fitness and biodistribution of edited HSPCs *in vivo* after NSG mice transplantation, as well as CAST-seq analysis to assess the safety of our editing process. We achieved about 90% of indel formation on HSPCs targeting the 4th intron of CX3CR1 and demonstrated the importance of the donor homology arm's length when optimising targeted integration on HSPCs, being the most successful the donor template leveraged the HMEJ (Homologous Mediated End-Joining). Furthermore, the GFP reporter cassette expression pattern skewed towards myeloid populations, with its expression peak observed in M1 macrophages. We also determined that the more differentiated HSPCs exhibited higher percentage and intensity of expression compared to the more primitive populations, such as CD34+low CD45RA-. Furthermore, we sorted GFP negative cells from either stem or linage-committed cells and differentiated them into M1 macrophages, we observed a switch-on phenomenon coherent with silent targeted integrations activating the cassette expression. The results demonstrated that the protocol was safe, as no off-targets events were detected after the CAST-seq analyses and no negative alterations were detected after NSGs transplantation. Importantly, we observed increased expression of our cassette in differentiated cells *in vivo* compared to hematopoietic stem cells (HSCs), particularly in CD11b cells, where CX3CR1 is expressed. Based on these results, we propose CX3CR1 4th intron as a safe harbour, holding an immense promise for potent paracrine cross-correction cell therapies without invoking genotoxicity in HSPCs. Moreover, we are currently conducting ChIP assays to shed light on the precise mechanism ruling over the cassette and CX3CR1 expression profiles.

P0643

Exploring mutation reversal via Prime Editing in hiPSCs-derived models: unveiling TAOK1 as a promising ASD therapeutic target through whole brain organoid scRNAseq analysis

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Autism Spectrum Disorder (ASD) is a prevalent neurodevelopmental disorder affecting over 1% of the global population, which its heterogeneity complicates understanding its causes and developing treatments. Despite identifying hundreds of ASD risk genes, none are found in over 2% of cases, underscoring the need for a personalised approach. Specifically, missense and truncated mutations in *TAOK1* have been identified in patients with severe neurodevelopmental delay, although its molecular and cellular functions remain largely unknown. The use of three-dimensional brain organoids sourced from human induced pluripotent stem cells (hiPSCs) have gained extensive usage as cellular models, as they can be derived from patients' cell lines; plus,

they promote the growth of diverse cell-type progenitor lineages and multiple neuronal subtypes within a tissue-like setting. Furthermore, the capability to introduce precise mutations to the genome of living organisms remains at the forefront of advancements in the life sciences and medicine. Concretely, base and prime editors can accurately introduce specific modifications in therapeutically significant cells without the need for double-strand breaks (DSBs). Prime editors allow the installation of almost any localised mutation, encompassing substitutions, insertions, and/or deletions of up to dozens of base pairs at designated DNA locations. This study seeks to investigate the effects of TAOK1 depletion by analyzing scRNAseq data from *wild-type* and K.O. forebrain organoids. These findings are crucial as they allow us to compare pathways that are differentially regulated due to TAOK1 depletion. Additionally, we can deduce the cell types where this gene is expressed, thus identifying the neuronal types most affected in patients with mutations in TAOK1. Furthermore, by utilising prime editing-based gene editing tools, we will explore various methods to introduce the target mutation, thereby assessing different strategies to achieve the highest efficiency in mutation installation. Hence, the ASD-related point target mutation will be installed in the hiPS cell line by using most different prime editing approaches, so the gene editing efficiency will be studied, allowing us to select the most optimal design to install the desired mutation. The significance of selecting the most efficient design lies in its potential for translation into a gene therapy capable of reversing the mutation across a maximal number of cells.

P0644

Superior editing efficiency and small size of CRISPR/hfCas12Max for gene and cell therapy applications

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The continued exploration of new Class 2 CRISPR-Cas systems in recent years has resulted in the identification of Cas12i. Though Cas12i holds promise as a tool for genome editing, enhancements in its efficiency and specificity are necessary for its effective utilization. Leveraging the HuidaGene – Platform for Rational Engineering of CRISPR-Cas Identification by Synergic Expertise (HG-PRECISE), a potent directed evolution system for both protein and guide RNA (gRNA), we have developed hfCas12Max, an engineered Cas12i, that recognizes 5'-NTN PAM with high editing activity and specificity in mammalian cells. Compared to Cas9 and Cas12a, hfCas12Max is more compact, making it an ideal candidate for single adeno-associated viral (AAV)-based delivery. Here, we assessed the applicability of hfCas12Max in creating gene editing therapies and cell therapies, incorporating multiple delivery methods such as AAV, lipid nanoparticle (LNP), and ribonucleoprotein (RNP). AAV is a commonly used gene-therapy delivery vehicle due to its proven efficacy, safety, and broad tissue targeting, including brain, skeletal, and cardiac muscle. After systemic delivery of a single all-in-one AAV vector that contained hfCas12Max along with a gRNA targeting the splice donor (SD) site of human *DMD* exon 51 to the Duchenne Muscular Dystrophy (DMD) mouse model, we observed efficient restoration of dystrophin expression. Histopathology and grip strength also showed significant improvement. Therapeutically acceptable editing efficiency in exon 51 SD was induced in a healthy wild-type non-human primate with a low dosage of AAV-hfCas12Max-gRNA without any

observed hfCas12max-related toxicity, indicating the relative safety of the AAV-hfCas12Max therapy. In further studies on Amyotrophic Lateral Sclerosis (ALS), AAV containing hfCas12Max and gRNA targeting the *ATXN2*gene considerably reduced toxic pTDP-43 protein aggregation to near-normal levels in the humanized *TDP-43*$^{Tg/Tg}$ mice, a widely used ALS mouse model. hfCas12Max notably alleviated astroglial and microglial inflammation in the motor cortex, restored motor function, and significantly extended the lifespan of the humanized *TDP-43*$^{Tg/Tg}$ mice. Moreover, LNP-mediated delivery of hfCas12Max mRNA and gRNAs led to a significant reduction in cccDNA, HBV-DNA, and hepatitis B surface antigen (HBsAg) levels in HBV-infected primary human hepatocytes (PHH). Two rounds of LNP-hfCas12Max system administration resulted in a sustained reduction of serum HBsAg and HBV DNA levels in the AAV-HBV mouse model. In addition, by administering hfCas12Max RNP targeting the *TRAC* gene in primary human T cells, we detected approximately 90% editing efficiency in CD3+ T cells, with cell viability remaining around 80%. In silico and genome-wide, off-target analyses indicated the high specificity of hfCas12Max. Our findings suggest that hfCas12Max, with its robust editing activity and high specificity, is a promising tool for safer and more effective gene and cell therapy treatments. Synthetic guide RNAs and purified nuclease for hfCas12Max are now readily available for CRISPR medicine developers through Synthego Corporation, USA, as part of a strategic partnership with HuidaGene to enhance the accessibility and efficiency of the CRISPR tools.

P0645

Enabling CRISPR-Cas associated research through guide RNA manufacturing solutions

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As CRISPR-based editing moves into therapeutic and clinical applications, it is critical to screen, select, and confirm activity of CRISPR guide RNAs (gRNA). This includes a feature to minimize unintentional genomic alterations from factors like manufacturing errors, cross-contamination, and other genotoxic effects. Leveraging experience in gRNA synthesis and manufacturing, we have done substantial work to ensure efficient on-target editing while reducing unintended events and toxicity. We have developed a method to characterize genome editing levels below 0.1% that are potentially attributable to low levels of cross contamination during manufacturing events. At the outset, our custom arrayed synthetic guide RNA libraries allow for accelerated guide screening and selection. Coupled with our chemically modified gRNAs, which include 2'-O-methyl and 2'-fluoro RNA nucleotides, gRNA libraries are an efficient way to execute CRISPR knockout screens. We enable a wide selection of guide formats and purifications, including HPLC-purified guides. Scale-up synthesis with HPLC-purified guides provide a stage-specific improvement over our current RUO sgRNAs which provide improved on target editing with a reduction in truncated products of synthesis. To support research and developmental stage-specific needs, we offer large scale RUO gRNAs, engineering run gRNAs, as well as cGMP guide RNAs manufactured in an ICH Q7 compliant facility. Our workflows and services include solutions for all applications, with CRISPR support, expertise, and documentation to meet the needs of the genome editing community target therapeutic applications.

P0646

Development of a Novel SMARTvector™ Multiplex shRNA Platform for Safer Cell Therapy Engineering

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Chimeric antigen receptor (CAR) T cell therapy represents a new era of cell-based immunotherapies, allowing for targeted killing of cancer, often blood cancers, expressing a specific antigen. Despite the success of several CAR T cell therapies achieving FDA approval, these therapies often suffer from T cell exhaustion, which can present as reduced cytolytic activity, increased expression of inhibitory receptors, and reduced proliferative capacity. These outcomes render the treatment less potent, pointing towards a need to develop more advanced and tunable methods of modulating CAR T cells to be more robust and reliable. Gene editing approaches such as CRISPR-Cas9 have successfully been used for disruption of genes such as PDCD1, an inhibitory receptor involved in CAR T cell dysfunction, to enhance CAR T cell performance *in vitro*. Despite the efficiency of CRISPR-Cas9, the number of targets that can be edited in a single sample is partially limited by increased cytotoxicity associated with DNA double-strand breaks (DSBs), thus limiting the therapeutic applicability. Here we present the SMARTvector lentiviral shRNA technology, a method for simultaneous multiplex gene knockdown in immune cells from delivery of a single expression vector. We show that up to eight shRNAs and an anti-CD19 CAR can be expressed after a single lentiviral transduction, thus providing a safer alternative of targeting multiple genes while maximizing CAR T cell engineering.

We developed the SMARTvector multiplex shRNA technology with multiple repeats of a novel patented microRNA scaffold, flanking artificial DNA sequences that target mRNA transcript(s) of interest for efficient gene knockdown. The vector design includes a tunable promoter and selection markers for specific targeted development. Here, an array of multiple shRNAs was generated in a single expression vector with efficacy measured by RT-qPCR to assess relative mRNA transcript knockdown or flow cytometry for assessment of functional protein knockdown.

We have demonstrated that multiple shRNAs can be expressed from a single expression vector, resulting in sustained expression and efficient gene knockdown in primary T cells and iPSCs. In addition to knockdown efficiency, we have engineered a vector with optimal linker length between each microRNA-based shRNA encoding region and evaluated position related impacts of each shRNA on the multiplexed cassette. We demonstrate that the SMARTvector multiplex shRNA technology is highly modular and adaptable, making it primed for use in a wide range of cell therapy applications, such as generation of allogenic CAR T cell therapies.

P0647

CRISPNA: A Novel Approach to Genome Editing and Diagnostic Applications

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CRISPR/Cas systems have emerged as transformative tools for scientists engaged in fundamental biological research, therapeutic development, and diagnostic applications. These systems rely on RNA molecules (crRNAs or sgRNAs) to guide Cas proteins to their intended DNA or RNA targets. While potent and specific, RNA molecules may exhibit instability in certain conditions and permit occasional mismatches during target binding. Peptide Nucleic Acids (PNAs), synthetically engineered oligonucleotides, offer superior affinity and specificity for binding complementary DNA and RNA when compared to conventional oligonucleotides. Also, their uncharged backbone endows PNAs with exceptional stability in biological fluids, as they resist degradation by proteases and nucleases. In this study, we introduce CRISPNA, a novel tool that combines the versatility of CRISPR-associated enzymes (Cas) with the robustness, stability, and specificity of PNAs. Our findings demonstrate that PNAs effectively guide different Cas proteins, such as Cas9 and Cas13, to their respective targets. Further analysis reveals that, in our initial designs, CRISPNA/Cas9's enzymatic activity is compromised, while CRISPNA/Cas13 functions optimally. Additionally, the CRISPNA/Cas13 complex exhibits sequence-specific cis- and trans-RNase activity in vitro. Notably, the in-vitro sensitivity of the CRISPNA/Cas13 system in its current configurations is 100-1000 times lower than that of the CRISPR/Cas13 counterpart. Remarkably, when electroporated, the CRISPNA/Cas13 ribonucleoprotein (RNP) demonstrates comparable efficiency to CRISPR/Cas13 in selectively degrading transcripts containing SARS-CoV-2 in HEK293T cells. In summary, we introduce here CRISPNA, a novel tool with a great potential, although PNAs designs for CRISPNA needs to be improved.

P0648

Delivery of Prime editing in human stem cells using pseudoviral NanoScribes particles

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Prime Editing can rewrite genes in living cells by allowing point mutations, deletions, or insertion of small DNA sequences with high precision. However, its safe and efficient delivery into human stem cells remains a technical challenge. Building upon *Nanoblades*, a non-coding virus-like particle system for the transfer of CRISPR-Cas9, we developed *NanoScribes* that encapsidate ribonucleoprotein complexes of Prime Editing. We identified key features that unlocked the potential of *NanoScribes*, including the use of multiple fusogens, the improvement of pegRNAs, their encoding by a Pol II system and the optimisation of Prime Editors. *NanoScribes* edited HEK293T with an efficiency of 68% at the *HEK3* locus with increased fidelity over DNA-transfection and support pegRNA-multiplexing.
Importantly, *NanoScribes* permit editing of myoblasts, hiPSCs and hiPSCs-derived hematopoietic stem cells with an editing efficiency up to 25%. *NanoScribes* is an asset for development of next generation genome editing approaches using VLPs.

P0649

Development of an Efficient Gene Writing System for *ex-vivo* cell therapy

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The development of effective, precise, and safe gene-editing technologies for ex vivo therapies, particularly in hematopoietic stem cells (HSCs) and chimeric antigen receptor T cells (CAR-Ts), poses a critical challenge. Current platforms face technical limitations and safety concerns, mainly related to cargo size constrains and off target activity of nucleases, impeding the realization of their full therapeutic potential. To address these challenges, FiCAT emerges as a groundbreaking gene-writing platform with the aim of providing a flexible and universal solution for safe editing of small regions and efficient, programmable editing of large genes in clinically relevant primary cells.

FiCAT is composed of a CRISPR-Cas nuclease and proprietary engineered transposase, with outstanding efficiency reached through rational protein engineering and an unsupervised variability generation approach. Over 400,000 variants are tested using a proprietary high-throughput screening system grounded in synthetic biology principles. In practical application, FiCAT RNPs had been produced and validated in T cells and HSCs using nucleofection for targeted integration of a minimal reporter. Our system compared favourably against existing systems like homology-directed repair (HDR) or homology-independent targeted insertion (HITI) using CRISPR-Cas9 alone. To enhance both efficiency and viability, electroporation is coupled with inhibitors targeting toxicity associated with double-stranded exogenous DNA. In addition, FiCAT therapeutic potential has been shown with the successful integration of CAR19 in a relevant locus (TRAC) for T cells. *In vitro* Functional validation of FiCAT generated CAR-T cells revealed comparable cytotoxicity with LV-generated CAR-T cells.

These results open up new possibilities with enhanced cargo size integration in HSCs and T cells, surpassing current gene editing techniques. This is of critical importance for both HSCs (i.e. correction of monogenic blood diseases) and T cells, since new generation of immunotherapies relies on targeting more than one epitope (Dual CAR) but also benefits from the inclusion of immunomodulators to enhance CAR-T cells efficiency and persistence.

FiCAT is designed to overcome precision and safety limitations in gene editing, streamlining the cell engineering process into a single step. Its potential to reduce costs and eliminate the need for viral vectors promises expanded accessibility to treatment. In summary, FiCAT represents a significant advance in gene editing, poised to enhance precision, safety, and cost-effectiveness in clinical applications.

P0650

Base editing mediated splice site disruption of *NR3C1* to induce glucocorticoid resistance in T cells

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Solid organ transplantation (SOT) is a life-saving procedure that can be compromised by infection and rejection. Immunosuppressive therapies are essential to maintain graft function and minimize rejection. However, these therapies also increase the risk of severe viral infections, such as CMV pneumonia and EBV-associated post-transplant lymphoproliferative disease. Virus-specific T cell therapy has shown promise as a potent treatment for these severe opportunistic viral infections and virus-associated malignancies in the SOT context. However, the immunosuppressive drugs used to prevent graft rejection, particularly glucocorticoids, can prevent optimal activation and induce apoptosis of adoptively transferred T cells, thereby limiting their efficacy.

To address this challenge, recent studies demonstrated that CRISPR-Cas9-mediated knockout of the glucocorticoid receptor gene (*NR3C1*) can effectively induce glucocorticoid resistance in virus-specific T cells without compromising their functionality in vitro. This suggests that gene editing could enhance the functionality of T cells in patients undergoing glucocorticoid-based immunosuppression. However, conventional gene editing techniques rely on inducing DNA double-strand breaks (DSBs), which can lead to unintended on-target mutations and potential larger genetic rearrangements.

Here, we investigated whether DSB-free base editing could be used to knockout *NR3C1* in T cells. We screened and identified single guide RNAs (sgRNAs) that may decrease *NR3C1* protein expression by disrupting splice acceptor or splice donor sites. To design sgRNAs for base editing, we utilized SpliceR (http://z.umn.edu/spliceR) to search for suitable sgRNAs within the *NR3C1* gene. Chemically-modified gRNAs and base editor mRNA were co-electroporated into activated primary human T cells. Sanger sequencing and editR analysis (baseEditR.com) were performed to assess the editing efficiency of the base editing at the DNA level. Three different sgRNAs were tested with different adenine base editors and cytosine base editors. We identified one sgRNA that enabled 100% efficient editing at the splice site on the DNA level when targeted with an 8th generation adenine base editor (ABE8.20m) fused to a Cas9 nickase. Compared to *NR3C1* knockout T cells generated by conventional CRISPR-Cas9 methods, *NR3C1* base edited T cells demonstrated comparable cytokine production and expansion rates. *NR3C1* base-edited and conventional Cas9-treated T cells displayed significantly improved survival in the presence of high doses of the glucocorticoid dexamethasone.

In summary, we have demonstrated that base editing can be used to introduce glucocorticoid resistance. In the future, these results may facilitate efficient multiplex editing of T cells to combine glucocorticoid resistance with other desirable features, thereby improving cellular activity in transplant patients.

P0651

Comparing the performance of different double-stranded DNA donor templates in CRISPR-Cas9 genome editing applications

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Genome editing using the CRISPR-Cas9 system can be harnessed to disrupt gene function, correct a pathogenic mutation, or insert an exogenous sequence into the genome. To facilitate insertion, a donor template with homology arms that flank the guide RNA (gRNA) target site must be introduced into cells along with the genome editing machinery. While synthetic oligonucleotides affecting SNP changes for small insertions can be quite efficient in mediating 'perfect' repair, knock-in of long (> 500 base pairs) double-stranded DNA (dsDNA) donor templates is generally inefficient. However, through innovations in donor DNA design, donor DNA modifications, and inhibition of proteins in competing double-strand break (DSB) repair pathways, we have observed additive and incremental improvement of dsDNA donor knock-in efficiencies via homology-directed repair (HDR). To this end, knock-in rates can be increased up to ten-fold by treating cells with small molecules and/or peptides that enhance HDR. Here we compare end-modified linear dsDNA templates (Alt-R™ HDR donor blocks, IDT) and circular dsDNA templates (Nanoplasmid™, Aldevron). In CRISPR knock-in experiments in K562, HEK293, and U2OS cells, Alt-R™ HDR donor blocks and the Nanoplasmid vector achieve similar knock-in rates in the absence of HDR enhancers, but in the presence of HDR enhancers, higher knock-in percentages are achieved with Alt-R™ HDR donor blocks, demonstrating up to 35% knock-in efficiency (with a 2 kb insert) in HEK293 cells with Cas9 mRNA and donor delivery achieved via electroporation in the presence of multiple HDR enhancers. However, in induced pluripotent stem cells (iPSCs), Cas9 mRNA and Nanoplasmid vector delivery by electroporation, in the presence of a single HDR enhancer, results in 35% knock-in efficiency of a 2 kb insert, indicating donor template performance may be cell-type specific. Furthermore, we assess how different gRNA target sites, donor template insert sizes, homology arm lengths, and modalities affect knock-in efficiencies of the aforementioned dsDNA templates in both immortalized and therapeutically-relevant cell types.

P0652

Enabling gene therapy with mbDNA™ (megabulb DNA) – A novel circular single-stranded CRISPR editing template

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1: Touchlight Ltd

Single-stranded DNA (ssDNA) is an excellent template for targeted genomic insertions via HDR. When used in combination with the latest genome editing technologies, its low toxicity and immunogenicity enable on-target insertional frequencies similar to those achieved by incumbent viral vectors. Viruses, although effective at transgene delivery and genomic insertion, present a significant biosafety risk due to their substantial cyto- and geno-toxicity. The packaging capacity of viral vectors is a further limitation to therapeutic applications that require large genomic insertions. Nonetheless, access to GMP-grade long ssDNA still presents a major challenge to adopting efficient and safe non-viral technologies for gene and cell therapy.

mbDNA™ is a long, synthetic, covalently closed ssDNA template for HDR developed by Touchlight, who manufacture GMP-grade DNA rapidly at scale. Utilising Touchlight's established enzymatic DNA production capabilities, the mbDNA platform can encode multi-kb sequences (overcoming the packaging constraints of viruses), and the elimination of fermentation processes and bacterially-derived sequences has further advantages for manufacturability and cellular toxicity. The mbDNA manufacturing process is the focus of a grant-supported scale-up program

at Touchlight, promising reliable supply at the scales and quality required for clinical applications and laying the groundwork for GMP supply.

As a synthetic ssDNA molecule, mbDNA has vastly improved toxicity ensuring a dose-dependent increase in knock-in and outcompetes existing DNA modalities. With 5-fold higher HDR efficiencies on average over double-stranded DNA, mbDNA generates significantly improved edited human primary cells yields. mbDNA has a consistent editing profile across blood donors and preserves the physiological profile of the edited T cell population at high DNA concentrations (480 nM), making it a safe and reliable CRISPR template of choice for autologous therapies.

A body of in-house and external evaluations by industry leaders has shown that mbDNA produces high rates of gene-length CRISPR-mediated insertion – used in conjunction with small molecule HDR enhancers, mbDNA achieves 70% knock-in in primary T cells, rivalling CAR-T viral vectors. Combined with faster recovery and better expansion post-editing, mbDNA-engineered cells more easily achieve the yields needed for clinical applications.

The covalently-closed nature of the molecule improves its stability, increasing its cellular half-life; head-to-head comparisons with commercially-available open ssDNA have demonstrated that mbDNA mediates 4-fold higher HDR frequencies and knock-in cell counts. mbDNA designs also incorporate binding sites for the associated nuclease ribonucleoprotein, enabling formation of a tripartite editing complex and enhancing transport of the mbDNA into the nucleus, overcoming a major bottleneck of non-viral technologies and further improving knock-in.

Ongoing evaluations by gene editing leaders are assessing mbDNA's performance in a multitude of clinically-relevant cell types, including T cells, iPSCs and HSCs. These promise to uncover mbDNA's full potential to revolutionise the gene and cell therapy field by improving the accessibility and efficacy of non-viral gene editing technologies, with data so far suggesting a highly adaptable platform.

P0653

Gene Editing induced Gene Silencing (GEiGS®) - A new technology to transform advanced therapies by programmable gene silencing

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Current cell therapies are burdened with challenges relating to efficacy, safety, and cost. Gene Editing induced Gene Silencing (GEiGS®) can be used be used to overcome these challenges by genetically engineering advanced therapies that respond to specific cellular conditions and/or disease states. GEiGS is a novel way of harnessing the RNAi pathway that works by introducing specific gene edits within endogenous redundant miRNA genes, to redirect their silencing activity towards new desired targets. GEiGS can enable silencing of both endogenous and exogenous targets and is highly amenable to multiplex engineering. In contrast to other approaches, GEiGS technology allows gene silencing that is programmable – linking silencing to cell identity or state; tunable – giving precise control of gene expression; stable – avoiding transient affects or epigenetic silencing; and specific – showing minimal loss or gain of function effects. These attributes individually and in combination provide a unique platform technology.

We have developed a flexible and efficient workflow for generating GEiGS-engineered therapeutic cells. First, our proprietary computational platform integrates data from miRNA expression profiles, genome annotations and target sequences. This system rapidly selects miRNAs, expressed in response to desired developmental and environmental cues, and recommends the necessary modifications for the efficient silencing of target genes. Second, engineered miRNAs are screened using high throughput pooled and arrayed approaches before they enter an automated cell engineering process. Finally, GEiGS-engineered cells progress into differentiation and in-vitro phenotypic validation for product development. These capabilities allow us to quickly iterate through design and product concepts, to facilitate broad technology implementation in autologous and allogeneic cell therapy products. We are exemplifying the use of GEiGS technology developing both iPSC-derived myeloid cells and primary T-cells for treatment of solid cancers, addressing many challenges with current therapeutic approaches.

In summary, Laverock's application of GEiGS technology enables safer, targeted cell therapies by engineering of human cells that exhibit silencing of the right genes, in the right cell, at the right level and at the right time.

P0654

Targeted gene insertion into Apolipoprotein C III (APOC3) locus achieves long term stable human FIX secretion in vivo in humanized APOC3 hemophilia B rat model

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Liver-directed AAV-based gene replacement therapies have shown clinical success in hemophilia. However, the therapeutic effect relies on the persistent presence of the AAV vector within cells, which remains as episomes. Since AAV vectors show a very low integration rate, if there is active hepatocyte proliferation or damage, therapeutic effects may disappear. This is a particular challenge for pediatric patients or hemophilia patients with other liver-related complications. Furthermore, as there are advancements in hemophilia care due to novel therapies, growing numbers of patients with hemophilia are living with age-related co-morbidities including cardiovascular diseases representing a new challenge for hemophilia treatment. To overcome these, we sought to insert a therapeutic gene into the APOC3 locus, a highly expressed hepatocyte-specific gene where its gain-of-function genetic variants were shown to worsen hyperlipidemia. We utilized the humanized APOC3 gene with FIX knockout rats to study the efficacy and durability of our strategy. We utilized neonatal rats where active hepatocyte proliferation is expected. Targeted in vivo human FIX insertion into human APOC3 locus showed sustained secretion of human FIX in serum up to 9 months post insertion. Corroborating with this, we observed corrected hemophilic symptoms including blood clotting ability and reduced spontaneous bleeding episodes of gene-inserted animals. Our results highlight that targeted gene insertion into the APOC3 locus supports its use as a novel safe harbor locus for sustained secretion of therapeutic proteins into serum and warrants further development of our strategy for not only hemophilia but also for indications with the requirement of life-long systemic supplementation of therapeutic proteins.

P0656

Engineering an AAV viral vector production cell line: SV40 T antigen locus removal in HEK293T via Mad7 genome editing

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The integration of the Simian Virus 40 T antigen (SV40 T Ag) and Neomycin resistance (NeoR) cassette into the Human Embryonic Kidney clone 293 (HEK293) generated the widely used HEK293T cell line. HEK293T cells display several advantageous attributes over HEK293, including increased growth kinetics and high transfectability. Oxford Biomedica (OXB) have an established HEK293T cell line which has been adapted to serum-free suspension growth conditions (HEK293T.1.65s). HEK293 cells generate high titres of both Adeno-associated viral (AAV) and adenoviral vectors (Ad). It has been widely demonstrated that HEK293T cell lines can achieve a higher productivity than the parental cell line HEK293. However, due to the theoretical safety concerns of the SV40 protein it is undesirable to use HEK293T cells for AAV and Ad production. The ideal platform for viral vector manufacturing would involve a cell line that preserves the beneficial characteristics of HEK293T cells, eliminates the SV40 T Ag, lacks antibiotic resistance cassettes, and efficiently produces high yields of AAV and potential other viral vectors such as Ad. OXB previously reported the generation of a clonal SV40 T antigen and NeoR free HEK293T cell line (OXBHEX1s.86) by performing Mad7-based genome editing. While the SV40 T Ag and NeoR protein expression was ablated from OXBHEX1s.86, DNA sequences from the integrated SV40 T Ag / NeoR locus remained. Here, we report a second round of gene editing on the OXBHEX1s.86 clone, utilising multiple crRNAs to target regions within the residual SV40 T Ag locus and adjacent genomic areas. This approach aimed to eliminate any remaining SV40 T Ag DNA sequences. Clones isolated from the edited cell population were screened for AAV9 production using a high throughput AAV9 screening assay as well as T7 PCR assays for assessing successful target deletions. Approximately 40% of the best clones were selected, expanded and re-screened at larger scale for AAV9 production, sensitivity to Geneticin and absence of PCR products to various amplicons across the SV40 T Ag locus region. The removal of the SV40 T Ag locus region was confirmed in top candidate clones using targeted Nanopore sequencing with further confirmation by Mass Spectrometry. The best candidate clone, OXBHEX2s.52, retains the fast-growing phenotype of HEK293T cells, lacks any DNA sequences from the SV40 T Ag or the NeoR cassette and produces viral vectors of high titre. Furthermore, this cell line has been successfully scaled up for AAV9 production at bioreactor scale. The results demonstrated higher titres compared to the existing process which uses a different HEK293 cell line, while preserving a similar product purity profile. In summary, the new OXBHEX2s.52 cell line offers a safer, more universal and streamlined way to produce life-changing gene therapy viral vectors.

P0657

Large-scale assessment of SpCas9 variants' efficiency, specificity, and PAM compatibility in cells

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Streptococcus pyogenes Cas9 (SpCas9) has been widely used for genome editing and exploited in curing human diseases. Heterogeneity of editing efficiency, concerns of potential off-targets, and restrains to few PAMs have been limited factors for broader applications of the technology. To overcome these hurdles, several SpCas9 variant with improved high-fidelity and broadened PAM sequences have been developed. Meanwhile and for unknown reason, these SpCas9 variants with improved specificity and PAM flexibility comes at the cost of reduced efficacy or specificity for some editing sites. Systematic assessments of these promising Cas9 variants with large target sites in cells are still needed, which will provide a useful guide for selecting the most appropriate SpCas9 variant needs to be brought up. Here we apply SURRO-seq, a high-throughput method based on surrogate gRNA cell library, to evaluate efficiency, specificity, and PAM compatibilities of 16 representative SpCas9 variants at thousands of sequences targeting human protein-coding genes with 2 cell lines. With the data collected from testing 12 variants with the NGG PAM using an on-target library, we observed that these variants perform differently and their overall efficacy aligns with previous studies. These extensive datasets will not only optimize the patterns of how these variants perform genome editing, but also aid in the development of deep learning models for selecting the optimal SpCas9 variant for given targets. This advancement will further propel CRISPR-based applications in both basic and clinical research, providing a robust framework for improved genome editing strategies.

P0658

Gene editing of ATM for the treatment of Ataxia telangiectasia in hematopoietic stem cells

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Ataxia telangiectasia (A-T) is a monogenic multi-system disease caused by inactivating mutations of the ataxia-telangiectasia mutated (ATM) gene, encoding the ATM protein kinase. A-T is characterized by progressive cerebellar degeneration, telangiectasia, immunodeficiency, radiation sensitivity, premature aging, and a predisposition to cancer. Life expectancy is severely compromised, and deaths are mostly linked to immunodeficiency and lymphatic cancer. Allogeneic hematopoietic stem cell (HSC) transplantation has therefore been explored in several patients to cure these two major disease aspects.

As an alternative, we set out to develop gene editing to treat A-T, based on knock-in of a codon optimized full-length ATM cDNA downstream of the transcription start of the mutated ATM gene in HSCs. As the full-length ATM cDNA is 9.2 kb large, exceeding the packaging capacity of most viral vectors, we chose to deliver the template for homologous recombination via 3 AAV vectors simultaneously, shortly after RNP complex electroporation. This editing strategy is optimal as a one-time treatment to correct all A-T causing mutations. We reached an efficiency of 3.5% in HSCs (CD34+ cells) and above 5% in lymphoid and myeloid cell lines. The lymphoid cell lines were generated from an A-T patient (AT006) or healthy control peripheral blood cells and were characterized as immature B-cells (CD10-, CD19+, CD20+, CD22-). When irradiated, AT006 cells showed a lower level of H2AX phosphorylation, as well as a slower recovery, compared to the control cell line. After irradiation, AT006 cells' return to a normal cell cycle was delayed as more cells were kept in G2 phase, compared to the control cell line. As the efficacy of the three AAV approach may not meet the therapeutic threshold needed, we have designed an alternative strategy to deliver partial ATM copies to correct only a percentage of all known A-T causing mutations by exon replacement of exons 10 to 13. Exon replacement was achieved by CRISPR-Cas9 mediated cleavage upstream and downstream of the targeted exons, providing the full exon and intron sequences by AAV vector transduction. This approach was tested in the same cell lines, with an efficiency ranging from 2% to 15%. Together, our data provides proof-of-concept on the feasibility of correcting ATM mutations by gene editing.

P0659

Investigation of the role of the DNA repair system in the biogenesis of circular DNA using CRISPR-C

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DNA fragments derived from chromosomes might persist in cells in a circularized form without reintegrating into chromosomal DNA. Due to the lack of centromeres, these DNA elements might segregate unevenly during cell division, resulting in diverse copy numbers in the derived cell population. Among these elements, extrachromosomal circular DNAs (eccDNA) comprise shorter sequences (a few kb), usually carrying non-coding genomic elements or only a fragment of a coding sequence. In contrast, extrachromosomal DNAs (ecDNA) range from hundreds of kilobases to megabases in size and may carry multiple genes, often oncogenes, representing one of the most frequent forms of oncogene amplification. Many recent studies using sequencing data from numerous cancer samples suggest the involvement of DNA repair system components in the formation of these circular DNA elements. Despite these studies, the role of many potential molecular actors has not been studied or confirmed in *in vitro* systems modeling eccDNA or ecDNA biogenesis. Here, we aimed to identify these key components through high-throughput analysis and provide direct experimental evidence of their role in the biogenesis of these circular DNA elements.

For this, we established a CRISPR-based knock-out library of 22 genes involved in DNA repair or DNA damage sensing. U2OS and HeLa cancer cell lines carrying an eccDNA sensing DNA cassette were used to establish gene knock-out lines. We induced the generation of eccDNA using our gene editing tool, CRISPR-C, and evaluated the gene knock-out effect on eccDNA generation and inversion. eccDNA generation induced GFP expression, and inversion formation resulted in mCherry expression. The fluorescent color changes in the cells were assessed by flow

cytometry. Our results show a strong reduction in DNA inversion events in the case of Ligase-4 and PRKDC gene knock-outs and a slight increase in eccDNA generation in the case of PRKDC gene knock-out.

To further evaluate the role of DNA-PKcs, a master regulator of non-homologous end joining (NHEJ), we applied its specific inhibitor to assess the effect of different levels of protein inhibition. Different levels of NHEJ inhibition differentially influenced the biogenesis of eccDNA but not DNA fragment inversion. Additionally, the reducing effect of DNA-PKcs inhibition on the formation of oncogene-carrying large ecDNAs was confirmed by ddPCR. In this study, we present the combination of our cell system and gene editing tool as a powerful setup to identify the molecular components of smaller and larger circular DNAs, highlighting the complex regulatory role of DNA-PKcs.

P0660

GeneAbacus: Swift PCR-free Gene Editing Validation Assay with Single Mismatch Precision for 96-well plates

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Introduction
Validating successful gene targeting is a critical step for CRISPR-based genome editing research. Traditional PCR-based methods for validation often require lengthy optimization to achieve actionable results. This translates into high costs associated with long-term maintenance of cell culture and model organisms. Moreover, highly tailored optimization efforts result in non-standardized protocols, making it challenging to achieve a standard gene editing validation practice. Here, we describe GeneAbacus, a PCR-free gene editing validation assay that does not require target-specific optimization, delivers in-house results within a day, and is compatible with crude DNA lysates and 96-well plate throughput.

Method
GeneAbacus utilizes padlock probes and Rolling Circle Amplification (RCA) chemistry to convert gene editing target sequences into quantifiable signals. The loss of wild type alleles and detection of specific edits are measured by digital quantification of signals obtained from edited and unedited cells. Multi-well throughput gene editing validation requires easy and fast genomic DNA extraction from cell lines and model organisms. We adapted the GeneAbacus reagent kit for crude DNA extraction from HEK293 & K562 cell lines and compared its sensitivity to column purified DNA. We also evaluated precision by quantifying a knock-out in HCT116 heterozygous clones. Finally, the specificity was demonstrated in a case study aimed at detecting a predicted deletion for pool validation and consecutive clone analysis. Results were compared against digital droplet PCR (ddPCR) and Sanger Amplicon Sequencing.

Results
GeneAbacus achieved similar sensitivity from crude DNA lysates as compared to column-purified DNA. Knock-out efficiency quantifications were also comparable between the two methods in a 96-well plate format. GeneAbacus demonstrated greater specificity than ddPCR in detecting

expected outcomes from a knock-out experiment, showing a stronger correlation with Sanger Sequencing than ddPCR assays.

Conclusion and Outlook
These results show that GeneAbacus performs gene editing validation with high accuracy and precision starting from both column-extracted DNA and crude DNA lysates. Its compatibility with 96-well plates make it an ideal tool for validating pools and performing clonal selections at a high throughput. The PCR-free nature of the assay removes the need for target-specific optimizations, and its ease of use and fast turnaround time represent a significant step towards standardizing gene editing validation and streamlining gene therapy research and development.

P0662

CA21113: Advancing Genome Editing for Human Diseases. Second Year Achievements of the GenE-HumDi COST Action Collaborative Network

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Recent advances in genome editing (GE) technologies have unveiled unprecedented opportunities for curing diseases through precise modifications of patients' genomes. Promising results have been achieved in animal models and ongoing clinical trials for genetic disorders, infectious diseases, and cancers. However, the insufficient integration of academic research into pharmaceutical companies' development strategies, limited interest in regulatory science, and the absence of established standards have hindered the broad application of these technologies for treating human diseases. The GenE-HumDi COST action is a fast-growing European network that brings together >250 scientists from 26 different countries and seeks to pull to pieces the barriers hindering the advancement of GE technologies for treating human diseases by fostering robust collaboration among pharmaceutical companies, academic institutions, scientific and regulatory agencies, and patient advocacy groups. Our primary goal is to expedite the clinical translation of GE technologies by addressing fragmented knowledge and establishing standardized procedures and guidelines. Through the creation of a synergistic network of GE groups, GenE-HumDi coordinates efforts to enhance current GE protocols, conduct safety assessments, optimize

delivery methods, promote clinical applications, and develop regulatory guidelines. This is accomplished through regular scientific meetings, development of training courses, and the sharing of data and materials, ensuring high scientific quality and reproducibility. A critical focus of our initiative is the establishment of standard operating procedures for GE safety assessments and delivery methods, as well as the comparison of different approaches to estimate off-target effects. Here, we present the core activities that GenE-HumDi has undertaken during the first two years of the Action. The first annual GenE-HumDi meeting in Granada, Spain (2023), and the second in Limassol, Cyprus (2024), have been instrumental in sharing recent advancements and enhancing communication among all relevant stakeholders, resulting in our first collaborative paper outlining the roles of GenE-HumDi members in advancing in the GE field. GenE-HumDi pays special attention to young researchers and to the geographical spread of knowledge into less research-intensive countries, and as such, regularly provides Inclusiveness Target Country (ITC) Conference Grants to Young Researchers and Innovators (YRI) working in ITCs and funds Short Term Scientific Missions (STSM) to foster collaboration among research groups within the network. With a special focus to train YRIs, GenE-HumDi also organizes Training Schools on different GE topics, as well as webinars led by top researchers in the field and showcasing the latest advancements in GE. Overall, GenE-HumDi has created a strong international and collaborative network of experts in the field and strives to accelerate the translation of gene editing technologies for the treatment of human diseases, through creation of knowledge exchange activities among the Action's members. Alongside showcasing its main outcomes, GenE-HumDi is looking forward to expanding its network and welcomes proposals for organizing future activities within the field of genome editing.

P0663

Rapid and simple diphtheria toxin-based selection of transgenic human cells via an engineered cell surface protein (selecDT)

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The generation of stable transgenic mammalian cell lines is vital for gene function studies and recombinant protein production. While current transfection techniques employing viral and non-viral vectors are efficient, the selection of transfected cells remains challenging. This is in part due to the inefficiency of antibiotic selection protocols in mammalian cells and the laborious process of cell sorting with fluorescent markers. To address this issue, we developed a selection method for transgene-expressing human cells using an engineered diphtheria toxin (DT) resistance protein, termed selecDT.

To easily generate human cell lines stably expressing recombinant proteins, we combined selecDT with non-viral transfections and the previously published cp36 integrase. We observed high integration efficiency of large DNA fragments into the unmodified human genome, with approximately 33% of cells integrating the donor plasmids. To rapidly select for cells expressing proteins of interest, we employed selecDT. We first showed that selecDT is expressed on the surface of modified human cells and provides robust protection from DT by inactivating its uptake receptor, thereby facilitating effective selection. Remarkably, in HeLa cells, selecDT achieved nearly 100% enrichment within one day of selection, in contrast to the extended selection periods required by traditional antibiotic-based methods. Finally, with the selectDT approach, complex

human proteins, such as ApoE and Fetuin A, were produced in HEK293 suspension cells achieving high yields.

We combined efficient transfection, integration, and selection approaches to establish a rapid and accessible method to create human producer cell lines. The novel DT resistance approach holds potential to speed up and simplify biotechnological processes and biomedical research.

This project has received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation program (grant agreement No 884505).

P0664

Optimizing CRISPR/Cas-Mediated CD-19 CAR knock-in efficiency, cell viability and cell expansion in human primary T cells using a cGMP-compliant electroporation platform

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Autologous cell therapy (ACT) harnesses the power of patients' own immune cells, engineered ex vivo to generate CAR expressing cells that are reintroduced into the patients as a living drug for the treatment of cancer. However, engineering of CAR T cells from autologous patients presents a multitude of challenges, including variations among different donors, low CAR expression and limited cell expansion. MaxCyte's clinical-scale flow electroporation technology efficiently delivers cell engineering tools in the form of DNA, mRNA, and protein into a wide variety of primary cells and stem cells. Here, we delivered multiple formats of donor DNA, linear dsDNA, and linear ssDNA from GenScript for in-frame GFP knock-in (KI) at the TRAC and RAB11A loci. Because variability of patients' cells is a significant challenge for autologous cell therapies, we investigated the effects of donor-to-donor variability on knock-in efficiency by performing CRISPR-Cas mediated non-viral engineering of primary human T cells from multiple T cell donors. Complexing Ribonucleoproteins (RNPs) with anionic polymer PGA (Poly-L-Glutamic acid) or treating T cells post-EP with Homology-Directed Repair (HDR) enhancing small molecules such as, M3814, Trichostatin A (TSA) and a commercial HDR enhancer further increased the GFP knock-in efficiency by 3-5-fold, depending on the small molecule used. PGA not only improved the KI efficiency but also improved cell viability by 20-40% post-electroporation, whereas HDR enhancers caused cell toxicity. We also delivered RNP complex for TRAC knock-out (KO) and donor DNA for in-frame CD19-CAR KI into primary human T cells and achieved ~40% KI efficiency. As with GFP KI, addition of PGA in RNP or HDR enhancing drug post-EP further enhanced the CD-19 CAR KI efficiency by 2-3-fold. In summary, we present data on KO/KI optimization, cell viability, cell expansion, cell phenotyping and potency assay post electroporation and clearly demonstrate that using MaxCyte's GMP-compliant electroporation platform and GenScript's reagents can engineer T cells from multiple donors with high efficiency that can be used for the treatment of diseases including cancer.

P0666

Controlling C-to-T editing with TALE base editors

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TALE base editors (TALEB) are fusions of a transcription activator-like effector domain (TALE), split-DddA deaminase halves, and an uracil glycosylase inhibitor (UGI). The C-to-T class of TALEB edits double strand DNA by converting a cytosine (C) to a thymine (T) via the formation of an uracil intermediate.

We recently developed and applied a strategy that allowed the comprehensive characterization of C-to-T conversion efficiencies within the editing window. This method also takes advantage of a highly precise and efficient TALEN®-mediated ssODN knock-in in primary T cells to assess how target composition and spacer variations affect TALEB activity/efficiency.

Specifically, we highlighted that the composition of bases surrounding the target TC may strongly influence the editing efficiencies. We also demonstrated that different TALEB scaffolds could be used to relax target sequence limitations (increasing the targetable sequence) or be used to decrease, or eliminate, bystander editing within the editing window (increasing specificity), overall allowing TALEB to be fine-tuned for a desired gene editing outcome.

We then applied a range of different technics to assess characteristics of nuclear genome editing. We first focused on on-target editing but also explored the possibility and risk associated with genome wide TALE dependent/independent binding and editing. By using an experimental model relevant for therapeutic application consisting in primary cells (PBMC and HSC) and TALEB mRNA vectorization, we demonstrated that single TALEB arm does not lead to detectable editing (detection limit: 0.1-0.2%).

We further applied hybrid capture assays to probe for off-target editing, in particular region of the genome that were previously highlighted in cells lines, and demonstrated, in our same relevant experimental setup, the absence of detectable editing (detection limit: 0.2%).

Altogether, the datasets obtained in this study enhanced our understanding of TALEB and permits the design of extremely efficient (high editing frequencies and edit purity) and specific (absence of TALE independent off site and very limited, if not absence of, DSB generation) TALEB, compatible with the development of future therapeutic applications.

P0667

Interchanging Cas enzymes, deaminases, and aptamer-guide RNA combinations to achieve optimal editing with the modular Pin-point™ base editing platform

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Base editing was first described in 2016 as a powerful tool to introduce precise genomic changes by avoiding DNA double-strand breaks and it has rapidly progressed to the clinic. However, its original Cas9 configuration is not able to address all genetic changes due to PAM limitations and the available deaminases. The uniquely modular Pin-point base editing platform is a three-component system consisting of either a nuclease-deficient or nickase Cas enzyme, plus an extended guide RNA with an aptameric scaffold, and an aptamer binding protein fused to a deaminase. These three components can be efficiently delivered as mRNA and synthetic sgRNA in primary T cells, iPSCs, and HSPCs to efficiently edit DNA targets of interest. Using the basic configuration of the system with a nickase S. pyogenes Cas9 (nSpCas9) and rat-APOBEC deaminase, the targeting capacity for base editing is limited due to the NGG PAM requirement of the nSpCas9 enzyme along with an editing window primarily targeting positions 4-8 within the protospacer for the rat-APOBEC deaminase. To target additional genomic locations, we have leveraged the modular nature of the Pin-point platform for an easily adapted “plug-and-play” approach in which we introduce different combinations of nucleases, deaminases, and sgRNAs containing varying aptamer positions within the gRNA scaffold. We have developed an arrayed screening platform for high-throughput characterization of these additional configurations as a way to demonstrate the unique editing characteristics of these novel systems. Using alternative Cas enzymes such as members of the Cas12 family and its T-rich PAM requirements enables editing at target sites that are not readily addressable using Cas9. Additionally, use of different species of deaminases such as the lizard derived Anolis-APOBEC enables editing with a wider editing window. When used in combination with different aptamer binding proteins and sgRNA scaffolds, we show that regions of the genome not previously attainable with previous configurations can now be efficiently edited. We also show that when using a dual aptameric approach, simultaneous editing with multiple deaminases can be performed in a single transfection as a way to edit two or more target sites and without the need for introduction of orthogonal Cas enzymes to deliver the different deaminases. This modular approach to base editing enables highly specific and efficient editing that can be custom tailored to fit specific cell and gene therapy programs and their unique editing requirements.

P0668

Development and validation of customized guide RNA design and efficiency prediction tools for the Pin-point™ base editing platform

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Base editors are a class of promising next generation genome editing technologies with the potential to both precisely correct disease-causing genetic variants and to safely knockout multiple gene targets simultaneously. The Pin-point base editing platform is a modular assembly of DNA binding Cas and DNA modifying deaminase components associated via an aptamer encoded in the sequence-targeting guide RNA (gRNA). A major challenge in the application of base editors in general is accurately in silico predicting the efficiency and specificity of editing at target sequences for a given combination of Cas and deaminase components to create a shortlist of possible gRNA designs for experimental validation. The modularity of the Pin-point base editing system allows the creation of a large number of configurations, that can vary in their PAM specificity, sequence editing preference and editing efficiency. To facilitate and accelerate the development of applications based on the Pin-point platform, we created a custom tool to design

gRNAs to target the gene of interest and to install base conversions, including those that would either install premature STOP codons or destroy splice sites to knockout the target gene. Additionally, we performed a massive parallel cell-based screen to analyse the editing activity of two different Pin-point base editor configurations with gRNAs targeting thousands of target sequences. We used the data obtained from the screen to construct models of the observed editing outcomes for each configuration. We applied these models to rank gRNAs designed to generate functional knockout at multiple clinically-relevant gene targets, including CIITA and PCSK9. After analysing the correlation of the in silico prediction with the cell-based performance of the gRNAs, we confirmed that the model predictions accurately correlate with the observed editing efficiency for the Pin-point base editing platform. The combination of the custom gRNA design tool and the predictive model led to the identification of a novel highly efficient gRNA able to knockout PCSK9 by disrupting a splice site, and we confirmed the predicted performance of other gRNA designs previously reported in the literature. Our gRNA design rules were informed using our broad cell-based performance dataset, creating reliable custom tools to prioritize gRNAs and select those with the highest editing efficiency.

P0669

Precise and efficient immune cell engineering via Pin-point™ base editing platform: insights from TotalSeq single-cell multiomic Analysis

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Many gene editing platforms, such as CRISPR-Cas9, rely on nuclease activity to introduce a targeted protein knockout through the introduction of DNA double-strand breaks (DSBs). While this revolutionary platform has been used in a wide array of cell and gene therapy applications and at varying stages of pre-clinical research up through recent FDA approvals, the technology does not come without risks. DSBs introduced by nucleases can lead to unwanted genomic alterations and cytotoxicity. These effects are magnified when more complex genomic perturbations, such as editing at multiple genomic sites, are introduced. Here, we demonstrate efficient multiplex gene editing using the Pin-point base editing platform as a safe and suitable technology for the creation of CAR-T cell therapies. The Pin-point platform can efficiently knockout four or more protein targets simultaneously with limited impact on cell viability in T cells and can also be utilized for a number of iPSC and HSPC therapeutic engineering applications. Through single-cell multiomic approaches, we can characterize the effect of editing with the Pin-point platform at the RNA and protein level. We see that the platform can efficiently edit multiple subpopulations of cells, for example, CD4, CD8, and dual CD4/CD8 positive T-cells, with high on-target efficacy and with no detectable off-target phenotypic impacts identified via interrogation of the whole transcriptome and a highly-multiplexed protein panel. From discovery to pre-clinical development, the Pin-point and TotalSeq platforms are well suited for engineering and characterizing next generation cell and gene therapies.

P0670

Reprogramming Large Serine Recombinases for Site-Specific Integration into Mammalian Genomes

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Accurate and efficient integration of large DNA sequences into specific genomic sites is essential for numerous medical, agricultural, and research applications. Current methods often rely on inducing double-stranded DNA breaks and relying on cellular DNA repair pathways such as NHEJ or HDR, which have their limitations. Serine integrases, such as Bxb1, offer a promising alternative as they can catalyze precise DNA recombination without generating free DNA ends. However, customizing these integrases for specific genomic targets remains challenging. We have employed directed evolution and rational engineering to generate Bxb1 variants capable of targeting defined genomic loci.

Through directed evolution of rationally designed libraries in bacteria, we successfully generated Bxb1 variants with high activity at defined genomic sites in the human genome. Furthermore, we show that that the activity levels achieved in bacteria translate in reporter assays in human cells. Finally, we demonstrate and confirm target integration at the DNA level in the selected genomic locus using our evolved variants.

Our study showcases the reprogramming of Bxb1 for integrating gene-sized DNA cargo into the human genome without requiring the prior integration of WT Bxb1 binding sites (attB). Efficient integration was demonstrated for a target site that differs in 17 out of 38 nucleotides from the WT attB target site demonstrating that we do not require pre-existing pseudo recombination sites with high homology to attB. A genome wide analysis with our proprietary search motif revealed flexible targeting across the whole human genome. This novel approach highlights the versatility of Seamless Tx Recombinase Programming technology, offering efficient and precise genome editing capabilities for a wide range of applications.

P0671

In vivo gene therapy of RDEB to restore collagen VII expression in epithelia using gene editing with adenoviral vectors in a mouse model

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6: FIBH12O 7: CIBERONC

Recessive dystrophic epidermolysis bullosa (RDEB) is a skin fragility disorder characterized by recurrent, slow-healing lesions affecting the skin, digestive tract and cornea. The underlying dermal-epidermal adhesion defect is caused by loss-of-function mutations in *COL7A1*, the gene

encoding collagen VII. We have previously demonstrated an NHEJ-based gene editing strategy for ex vivo gene correction in cells from RDEB patients, based on the removal of exons carrying mutations in the collagenous domain of COL7A1. Building on this, we have implemented tools and strategies for in vivo therapy of RDEB using NHEJ-based gene editing to restore collagen VII expression in a hypomorphic mouse model of RDEB. This hypomorphic model has an FRT-flanked neoR cassette inserted between exons 2 and 3 of the *Col7a1* gene, causing splicing aberrations, frameshift, and premature stop codon. This results in low levels of functional collagen VII in the basal membrane zone of stratified epithelia, RDEB phenotype, and high perinatal lethality. We used helper-dependent adenoviral vectors (HD-AdV) to express CRISPR targeting FRT sequences, to remove the neo cassette and restore expression of functional Collagen VII. We first tested this strategy in neonatal *Col7a1* hypomorphic skins grafted onto immunodeficient mice to circumvent the high neonatal mortality of these mice. The HD-AdV vector embedded in a fibrin matrix was delivered to full-thickness wounds on grafted skins. After complete healing of the treated wounds, we detected the presence of collagen VII in the basal membrane zone limited to the treated areas. After assessing the feasibility of *in vivo* correction in the skin, we administered the adenoviral vector to neonatal mice by injection through the temporal vein for systemic delivery. Immunohistochemical analysis of samples collected one week after a single dose injection showed widespread restoration of Collagen VII expression in stratified epithelia associated with a reduction in blister formation. These results demonstrate the use of viral vector-delivered CRISPR for the NHEJ-based correction of COL7A1 to alleviate the RDEB phenotype.

P0672

Toward gene therapy for Rett syndrome: preclinical evaluation in patient-derived cells of gene editing as a therapeutic approach

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Approximately 95% of patients with Rett syndrome (RTT; OMIM#312750) present pathogenic variants in the X-linked gene *MECP2*. Reactivation of *Mecp2* in symptomatic KO mice can revert disease phenotypes, suggesting that RTT is not irreversible. Classic gene replacement therapy is currently being evaluated but it is complicated by the need of finely tuning *MECP2* expression, since both over- and under-expression result in disease. Effective therapies are not currently available and gene editing based on CRISPR/Cas9 combined with Homology Directed Repair appears an appealing option for the development of new therapeutic approaches, since it allows maintaining the endogenous regulatory framework. To validate its applicability for *MECP2* hotspot variants we established an international consortium that was funded from the EU in 2021. In particular, we tested the approach for four *MECP2* mutation hotspots, namely c.473C>T (p.(T158M)), c.502C>T (p(R168X)), c.763C>T (p.(R255X)), c.916C>T (p.(R306C)). To this aim, we obtained cellular and mouse models in order to demonstrate the safety and effectiveness of *MECP2* gene editing in reversing Rett phenotype, both *in vitro* and *in vivo*. Next Generation Sequencing analysis of edited cells has demonstrated efficient editing for c.473C>T - (p.(T158M)) and c.502C>T (p(R168X)). To further validate efficacy and safety in a more complex context, experiments will be performed in brain organoids and *in vivo* in available KI mouse models. Specifically, we are focusing on the c.502C>T (p(R168X)) variant, since sequence homology will allow employing the same sgRNA employed in patient cells. In the meantime, we are working to identify molecular readouts for validating the functional effectiveness of editing. Since mTOR pathway has been found downregulated in RTT animal models, we evaluated the expression and phosphorylation of 2 proteins downstream of mTOR pathway: p70 ribosomal protein kinase (p70S6K or p70) and S6 ribosomal protein (S6). Our results confirm a dysregulation of the pathway, with reduced phosphorylation of both proteins in both neuronal precursors (NPC) and neurons with mutations in *MECP2* gene.

P0673

Minimal activation of the p53 DNA damage response by a modular cytosine base editor enables effective multiplexed gene knockout in induced pluripotent stem cells

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Precise genome editing of induced pluripotent stem cells (iPSC) holds great promise for engineering advanced cell therapies. CRISPR-Cas systems have been widely adopted in genome engineering applications, however their dependence on genotoxic DNA double strand breaks (DSBs) presents challenges in hypersensitive iPSCs, including the selection for defective DNA DSB responses. Base editors are capable of both modifying and ablating gene function without generating DSBs making them an attractive solution in iPSC engineering applications. Here we report efficient and durable target knockout and substantially improved cell viability and expansion with a cytosine base editor assembled using the Pin-point™ platform compared to SpCas9. The cytosine base editor minimally activated p53-mediated DNA damage signalling independently of the number of simultaneous edits installed. By contrast, multiplexed editing with SpCas9 lead to high levels of p53 signalling and an associated reduction in editing efficiency. While transient inhibition of the p53 DNA DSB response enhanced SpCas9 multiplexed gene editing efficiency this was not required to achieve maximal base editing efficiency. Multiplexed editing of iPSCs with Pin-point base editors therefore both enhances the efficiency of genome

engineering processes and substantially reduces the risk of selection for defective DNA damage responses inherent to DSB-dependent CRISPR-Cas systems.

P0674

A mutation-agnostic genome editing approach for Autosomal Dominant Polycystic Kidney Disease (ADPKD) using CRISPR-Cas9 delivered using proprietary kidney tropic lipid nanoparticle

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Autosomal Dominant Polycystic Kidney Disease (ADPKD) is a hereditary condition caused by mutations in the *PKD1* (85% cases) or *PKD2* (13% cases) genes, which encode polycystin-1 and polycystin-2 proteins, respectively. This condition is characterised by the formation of fluid-filled cysts within the kidneys, which arise from renal tubules and can lead to end-stage renal disease (ESRD). *PKD1/PKD2* genes are significantly repressed in ADPKD and lead to cytogenesis. In various pre-clinical models and in humans with ADPKD, *PKD1/PKD2* gene re-expression has displayed attenuation as well as reversal of cyst formation proving this strategy to be of therapeutic potential. As 85% of ADPKD cases are caused by *PKD1* mutations and there are more than 800 pathogenic mutations in *PKD1*, we are pursuing a mutation-agnostic approach that can solve for all the mutations of *PKD1* rather than the conventional single-target/mutation specific therapies for ADPKD. We employ Helex's proprietary Epic-Cure™ platform, which uses 3D genome architecture and epigenetics of target and disease-specific cells to design gRNAs in conjunction with the molecular scissor CRISPR-Cas9, to edit the mutation agnostic target that regulates *PKD1* gene in the most effective, precise and safe way.

One of the most crucial challenges for developing a therapeutic for renal indications is the delivery to the kidney cells. Our proprietary lipid nanoparticles (LNP) are designed to facilitate kidney delivery of the gene editing cargo. We have successfully demonstrated editing of the target using the LNP-CRISPR Cas9 machinery in ADPKD patient-derived cells (WT9-12 and WT9-7 cells) and sequence confirmed it. Additionally, a significant four-fold increase in *PKD1* mRNA levels was observed after editing, where about two-fold is needed for therapeutic benefit. An increase in PC1 levels and changes in other downstream cystic pathways such as pCREB pathways post target editing was evidenced by reverse transcription polymerase chain reaction (RT-PCR) and western blotting techniques. Significant kidney tropism was observed for our proprietary LNP via tail vein injections in BALB/c mice using a DiR dye both in *in vivo* and *ex vivo* imaging. In addition, these lipid nanoparticles displayed cell-specific uptake in kidney epithelial cells when they were encapsulated with mCherry mRNA and delivered using multiple delivery routes such as renal artery injection. Our mutation-agnostic, single-therapy strategy targets the majority of the ADPKD patient population. Harnessing Helex's proprietary kidney-tropic lipid nanoparticle and the Epic-Cure™ drug design platform, this approach holds promise in halting the disease and reversing its phenotype. This goes far beyond current treatments, which focus on managing symptoms and slowing the disease's progression.

P0675

CRISPRon/off: combined Cas on/off-target design

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The design tools for CRISPR are rapidly evolving, trying to keep up with the latest developments in experimental CRISPR methods. The design of an optimal gRNA is a job in two parts. The first part is the selection of *efficient* gRNAs that can generate the desired change, while the second part is the search for potential *off-targets* for the efficient gRNAs. Off-targets for CRISPR/Cas9 mediated edits are of critical importance for both experimentalists and clinicians in determining potential side effects of an experiment or clinical treatment. Here we present a combined workflow for on/off-target assessment based on our CRISPR/Cas webservers, **CRISPRon**, which is based on the currently best model for on-target efficiency of Cas9 (with Cas12a to appear soon), and **CRISPRoff**, which is based on a versatile energy model for gRNA off-targets capable of competing with the best available deep learning methods for Cas9 (off-target assessment for Cas12a will appear soon). A Cas9 base editor on-target design tool is under development as well. Find all the tools via https://rth.dk/resources/crispr.

P0676

Synthetic, enzymatically produced DNA and non-viral delivery systems for gene editing applications

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Long lead times associated with high-quality, GMP grade DNA is a major limitation in the production of mRNA encoding nucleases, and DNA integration templates for use in gene editing applications. Further, complex sequences, such as long homopolymeric sequences including long polyA tails, are difficult to propagate in bacteria. 4basebio has developed a proprietary, scalable, fully enzymatic synthesis process for the production of linear DNA constructs via our Trueprime™ amplification technology. DNA yields of 1 g/L are achieved, several orders of magnitude higher than those of plasmid fermentation processes, enabling a small footprint using benchtop equipment. The process is size and sequence independent, facilitating large scale production of GMP grade linear DNA with high yield and purity in less than a week. In contrast to plasmid DNA, contamination from endotoxins or host proteins is avoided, and amplification of bacterial sequences, such as antibiotic resistance genes, is not required.

4basebio offers several solutions for gene editing applications. Synthetic double-stranded open-ended (op/oe)DNA™ can be used as a starting template in the IVT production of Cas9 mRNA without the need for enzymatic linearisation. Meanwhile, single stranded (ss)DNA™ and oeDNA™ can both be used as an integration template for HDR strategies. In addition, Hermes™, 4basebio's proprietary non-viral delivery system, exploits a cationic ligand and traditional lipids to drive encapsulation of a range of payloads, including synthetic DNA, mRNA, RNP complexes, or a combination thereof. Hermes™ nanoparticles have demonstrated comparable bioluminescence to commercial LNP formulations when delivering firefly luciferase mRNA intramuscularly to mice and, exceed commercial LNP formulations for hpDNA™ delivery.

Here, we combine our synthetic DNA, mRNA and Hermes™ platforms for gene editing applications. When encapsulating Cas9 mRNA and gRNA, Hermes™ formulations routinely achieve high knockout efficiency and high cell viability of GFP in HEK293 cells that stably express GFP, significantly greater than CRISPRMAX reagent. Further, Hermes™ formulations encapsulating Cas9 mRNA/gRNA and ssDNA™ as a HDR template have been successfully employed to introduce a 4-point mutation, switching eBFP2 into eGFP via the conversion of just three amino acids. Likewise, oeDNA™ used as a long HDR template has achieved superior integration efficiency than plasmid DNA in a multitude of cell lines using a gRNA targeting the human ACTINB gene. Importantly, a reduced DNA template size, owing to a lack of bacterial backbone, allows for a copy number advantage over plasmid DNA, facilitating reduced DNA doses and improved toxicity profiles. Finally, 4bb ssDNA constructs consistently perform comparatively to commercially available templates when delivered via electroporation in primary cells, with increased viability. The combination of 4basebio's synthetic DNA platform and non-viral delivery system can accelerate clinical development of gene editing applications.

P0677

Development of CRISPR/Cas9 Gene Therapy for RYR1-Related Myopathies

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The Ryanodine Receptor Type 1 (RyR1) is an essential calcium channel for skeletal muscle contraction. Mutations in the *RYR1* gene are responsible of rare diseases called "RyR1-related myopathies," which lead to muscle weakness in affected individuals. Currently, no cure exists for these conditions. One of the main obstacles in developing gene therapy for RyR1-related myopathies is the extensive number and widespread distribution of mutations within the *RYR1* gene. Additionally, gene replacement therapy is not feasible due to the large size of the *RYR1* gene.

To address these challenges, we have developed a versatile approach to restore RyR1 function regardless of the specific patient mutation. Our strategy involves the targeted deletion of the pathogenic allele using CRISPR-Cas9 technology. We have demonstrated the efficiency of this approach in immortalized myoblasts derived from a patient with a dominant *RYR1* mutation. The next step is to apply this methodology to a mouse model.

For the in vivo proof-of-concept, we optimized both the delivery method and the efficiency of the guide RNAs (gRNAs). First, we explored the best route of administration to achieve high levels of transgene delivery in muscle tissue with optimal specificity. We used an AAV vector with strong muscle tropism, known as MyoAAV, which expresses Green Fluorescent Protein as a reporter. Next, we performed *in vitro* screening of various pairs of gRNAs targeting the pathogenic allele and we selected the most effective candidates for *in vivo* gene editing. Our project will pave the way for applying a novel strategy of genome editing to treat RyR1-related myopathies.

P0678

High-efficiency homology-directed insertion into the genome using ARCUS nucleases

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There are now many gene editing tools poised to enter the clinic, representing varied options for eliminating or correcting mutations. Although there is a wide range in capabilities, several limitations persist. For instance, base editors are accomplished at switching out a particular base but cannot make all forms of base changes, nor do they support large insertions or deletions. Stimulating gene editing by homology directed repair (HDR) has the potential advantage of being able to accomplish any type of edit as defined by the repair template, but gene editing enzymes that support efficient HDR are a relative rarity.

Homing endonucleases are a family of proteins that have evolved to safely insert a copy of their gene into a genome using HDR. ARCUS nucleases are engineered from the homing endonuclease I-CreI and have programmable sequence specificity. The following work demonstrates that ARCUS nucleases maintain the ability of a homing endonuclease to drive efficient DNA repair by HDR. In T lymphocytes, we observed that HDR-mediated insertion of a DNA template could be achieved in greater than 85% of cells when the template was delivered by AAV6. Following cleavage of its DNA target site, ARCUS nucleases leave behind a 4 base pair, 3' overhang. We demonstrated that removing this overhang (with exogenous exonuclease activity, or by introducing a DSB with a blunt-cutting endonuclease) resulted in greatly reduced integration efficiency. We further explored the importance of the unique cut generated by ARCUS, as well as the impact of homology arms and the contributions of the cellular DNA repair machinery, as they pertain to genomic integration.

Using HDR, we demonstrated ARCUS's ability to support the full range of currently understood DNA editing approaches including all combinations of base changes, introducing small, specific deletions, small and large insertions, and even the ability to replace large segments of DNA within the genome. Collectively, we show that the uncommon enzymology of ARCUS nucleases drives high rates of HDR and that this can be leveraged to execute a multitude of highly precise gene editing functions for therapeutic benefit.

P0679

LinQURE®: A novel AAV gene-silencing platform for multi-transcript targeting towards the treatment of complex genetic disorders

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Innovation in Adeno-Associated Virus (AAV)-based gene therapy, focusing on both capsid and cargo, is crucial for addressing the vast landscape of genetic disorders. Our proprietary miRNA-

based miQURE® technology enables precise targeting and downregulation of transcripts, mitigating the progression and severity of these diseases. Enhancing this approach, we developed the linQURE® platform by concatenating optimized synthetic miRNAs into a single construct. This advanced method improves targeting efficiency for specific transcripts and introduces multi-targeting capabilities, enabling our gene therapy products to tackle complex multigenic conditions. This poster demonstrates the versatility of linQURE® technology through single-transcript, multi-transcript, and sense-antisense transcript targeting strategies. Our data show that linQURE® facilitates the expression of multiple synthetic miRNAs, resulting in efficient downregulation of disease-causing targets both *in vitro* and *in vivo*. These findings underscore the therapeutic potential of linQURE® for a broader range of genetic disorders than using a single miRNA.

P0680

High efficiency complex gene editing of hard-to-transfect primary cells using MaxCyte electroporation in combination with Synthego sgRNAs

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Cell-based therapeutics using engineered primary cells hold great promise for treating a variety of diseases, including cancer and autoimmune disorders. Increasingly, researchers are using complex CRISPR gene editing strategies to develop highly engineered cell therapies with greater potency, more favorable safety profiles, and better manufacturability than previous generations of cell therapies. These CRISPR-based editing strategies often involve introducing multiple edits – including both knockouts and knockins – performed either in sequence or simultaneously to generate highly engineered cell products tailor-made for a specific disease of interest.

As the complexity of genome engineering increases, so do the challenges associated with developing efficient, scalable, and reproducible manufacturing processes. Issues arising from cell loss, low transfection efficiency, and high donor variability are often exacerbated as engineering complexity rises, increasing the risk of manufacturing failure and potentially compromising product quality.

To simplify and de-risk complex gene editing workflows, we have developed a versatile cell engineering process that combines MaxCyte electroporation with Synthego sgRNAs to enable a wide range of CRISPR-based gene editing strategies in primary human cells. Here, we show that using MaxCyte electroporation to deliver Synthego CRISPR gene editing tools as part of a cell therapy manufacturing process enables efficient and reproducible gene editing across a variety of cell engineering workflows. We show that this cell engineering strategy is suitable for use in many of the hard-to-transfect primary human cell types – including primary human T cells, NK cells, keratinocytes, and macrophages – that are increasingly being used in the development of next-generation cell therapies.

P0681

Insights of CMC, Quality & regulatory for mRNA-based gene editing program – A case study and lessons learned from early phase development to final GMP manufacturing for IND application

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In the rapidly evolving field of mRNA-based gene editing, the development and manufacturing of such innovative therapies demand a comprehensive understanding of CMC processes. ReciBioPharm has successfully delivered multiple GMP programs in this field from early phase prototype, process and analytical development to final GMP manufacturing. Leveraging the unique experience, this abstract introduces a case study that delves into the CMC best practices and invaluable lessons learned in the journey from sequence to IND for a Gene Editing Program.

The presentation will provide an in-depth exploration of the following key aspects:

1. Development and Manufacturing Considerations: We will spotlight the critical points along the road in plasmid DNA, drug substance, and drug product process and analytical development and GMP manufacturing.
2. Regulatory and Quality Assurance: We will address the regulatory hurdles and how to navigate in the early phase landscape while still ensuring patient safety and successful IND submission.
3. Lessons Learned and Future Perspectives: This case study reflects on the challenges faced, setbacks encountered, and the adaptation of strategies during the entire development and manufacturing process. Insights gained from real-world experiences will offer valuable guidance to sponsor companies.

It serves as a vital resource for CMC stakeholders, offering a roadmap for success in bringing mRNA-based gene editing therapies from sequence to IND.

P0682

Cas9 antigenicity – addressing adaptive immune response activation

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CRISPR/Cas9-based gene editing brings hope for treatment of rare genetic diseases. Overshadowing the promise, emerging reports of pre-existing and treatment induced host immunity to the prokaryotic Cas9-endonuclease challenge the feasibility of *in vivo* therapeutic gene editing. Hence, to address the Cas9 antigenicity we measured lymphocyte response to Cas9-based stimulation and observed type II interferon response indicating of priming and activation of Cas9-specific T-cell response. In therapeutic context, the formation of cellular host

immune response against the core component of therapeutic modality may results in rejection of the targeted cells, potentially compromising the treatment outcome. Hence, modelling Cas9 antigenicity and understanding how to mitigate it is crucial for versatile utility of therapeutic gene editing.

P0683

Development of off-the-shelf CAR-macrophages derived from pluripotent stem cell-derived myeloid cell lines

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One of the challenges of chimeric antigen receptor (CAR)-T therapy is its limited efficacy in solid tumors, and CAR-macrophages are expected to be a promising antigen-specific therapeutic alternative that could overcome this problem. The development of off-the-shelf products using pluripotent stem cell (PSC)-derived immune cells with CAR construct is an attractive strategy to reduce the cost of CAR-immune cell therapy. However, differentiation into macrophages from PSCs is still costly and time consuming. In this context, PSC-derived immortalized myeloid cell lines (MLs), capable of unlimited proliferation, have great potential as a resource for immunotherapy. Here, we established anti-HER2 CAR-macrophages derived from human pluripotent stem cell (PSC)-derived myeloid cell lines (PSC-MLs). PSC-MLs, an immortalized monocytic cell line, can expand exponentially in M-CSF dependent manner and differentiate into macrophages. The resultant PSC-ML-derived CAR-macrophages show potent antigen-specific killing activity via phagocytosis in vitro against tumor cell lines expressing HER2 antigen and suppressed tumor progression in vivo. Our results provide an effective, allogenic and off-the-shelf cellular resource for adoptive cellular immunotherapy against solid tumor.

P0684

Design of inhibitory domains for CAR-T cell regulation

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CAR-T cell therapy has emerged as a revolutionary approach in cancer treatment, demonstrating remarkable efficacy and the potential for long-term remission. However, this powerful therapy is not without limitations. This includes T cell exhaustion and side effects that may occur in therapy: Cytokine release syndrome (CRS), a severe inflammatory response, and CAR-T cell-induced neurotoxicity, which can lead to confusion and even seizures. Current treatment for these side effects relies primarily on reactive measures using immunosuppressive drugs. We propose a proactive strategy for managing CAR-T cell activity to manage cell exhaustion as well as side effects. By introducing regulatory mechanisms within the CAR-T cells, we aim to achieve a more

precise and reversible control over function. This approach holds the promise of enhanced therapeutic efficacy while minimizing the risk of debilitating side effects. To achieve precise control over CAR-T cell activity, we designed and tested several inhibitory domains. These domains are inspired by natural T cell signalling mechanisms and function by regulating CAR-T cell signalling pathways. To introduce external control, we fused the inhibitory domains and CAR with chemically inducible dimerization domains. This design ensures that CAR-T signalling is only inhibited upon the addition of a specific small molecule from outside the cell. In the absence of the small molecule, all constructs induced cell activation. The level of activation differed between constructs, depending on the orientation of heterodimerization domains. We achieved complete inhibition of cell activation upon the addition of small molecule regulator, while the control construct exhibited no change in activation. In essence, this approach allows for the precise and tunable regulation of CAR-T cell activity, offering a promising strategy for safer and more effective CAR-T cell therapy. Future research will focus on optimizing the inhibitory domains to ensure the effectiveness and safety of this approach.

P0685

Addition of a TLR costimulatory domain enhances the activation of CD19 CAR T cells

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Chimeric antigen receptor (CAR) T cell therapy is a type of immunotherapy that has recently emerged as an effective treatment against several hematological malignancies. Intracellular signalling domains of genetically modified CARs derive mainly from T cell receptor and their costimulatory receptors. In addition to the conventional signalling molecules, Toll-like receptors (TLRs) can also function as costimulatory signals to enhance T cell activation, proliferation and survival. Therefore, TLR-derived signalling elements could be potent costimulatory domains in the structure of CAR T cells. In our study, we developed anti-CD19 CARs that besides the 4-1BB costimulation domain also contain TLR activation domains. We designed and generated various CAR constructs with specific elements of adaptor protein MyD88, TRIF, and additionally some constructs with truncated TLR4. First, we made the selection of our CARs by measuring hIL-2 production from Jurkat cells that had been previously electroporated with plasmid constructs and co-cultured with target CD19 positive cells. Constructs that generated higher activation compared to second-generation CAR were selected for further experiments. Human T cells transduced with selected CAR construct were found to be highly potent at inducing hIL-2, hIFN-γ, hTNF-α and cytotoxicity when co-cultured with several CD19 positive tumor cell lines. The analysis with the CD19 negative cell line did not show any cytokine secretion. No significant difference in immunophenotype was observed between our CAR T cells and second-generation CAR T cells. Finally, the efficacy of our CARs was evaluated in vivo using an NSG mouse model. Our findings suggest that TLR signalling could enhance the activation of the CAR T cells and that the additional TLR costimulatory domain did not induce constitutive activation.

P0686

Uncovering functional heterogeneity of switchable UniCAR-T cells with a novel microfluidics-based single-cell immune cell killing analysis

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CAR-T cells have shown unprecedented clinical success in treating haematological malignancies, but severe adverse effects may appear in the clinical setting. To address such potential safety concerns, switchable CAR-T strategies have emerged as a powerful tool to improve the controllability and mitigate potential toxicity of these therapies. In this study, we perform a single-cell functional characterization of the adapter UniCAR-T cell platform, which incorporates a cross-linking molecule named Target Module (TM) as a molecular safety switch to regulate CAR-T function.

While serial killing by CAR-T cells has been reported, it is still not known: (i) how many tumor cells can be killed by a single CAR-T cell, (ii) if all CAR-T cells are able to perform serial killing or (iii) killing at all, and (iv) the variation of activity over time. Addressing single-cell functional heterogeneity of distinct CAR-T subpopulations is crucial to understanding the clinical potential of novel cellular immunotherapies. However, assessing this diversity is technically challenging.

Here, we leverage a novel microfluidic platform to investigate the complex interplay between UniCAR-T, tumor cells, and the TM by using single-cell functional profiling to identify rare serial killers. Our co-culture array microfluidic technology creates over 19.000 miniaturized tumor-immune co-cultures on each array plate. Microwells are imaged, tracked, and analyzed using a combination of robotics and AI-powered image analysis to minimize hands-on time.

We evaluated the immune cell killing potential of UniCAR-T cells directed against $CD33^{+}$ HL-60 tumor cells using a scFv-based anti-CD33 TM. We generated thousands of miniaturized co-cultures at various effector-to-target (E:T) ratios and analyzed bulk immune cell killing in the presence or absence of varying concentrations of anti-CD33 TM for 24 to 48 hours. Our results revealed significant specific killing activity of UniCAR-T cells in the presence of anti-CD33 TM against HL-60 cells, and unveiled the killing kinetics. Such kinetic analyses reported bulk cytolytic activity of UniCAR-T cells after 6 hours of co-culture, without observing substantial earlier activation. We then leveraged our microfluidic technology to interrogate the function of hundreds of individual UniCAR-T cells by directly measuring tumor cell killing. Given that the modular design of the UniCAR system allows precise control of immune cell-mediated tumor killing, characterizing the serial killing capabilities of UniCAR T-cells is of particular interest. Remarkably, our single-cell analyses revealed distinct functional subpopulations with varying degrees of activation over time, uncovering the existence and frequency of a subset of highly active UniCAR-T cells, named ‘serial killers’, capable of killing at least three tumor cells. This subset accounted for over 10% of the total UniCAR-T population at the latest time points of the analysis.

In summary, our analysis integrating single-cell and kinetic functional profiling provided a detailed characterization of the killing capabilities of functionally distinct UniCAR-T subpopulations and identified a rare subset of highly potent ‘serial killers’ with enhanced tumor killing potential. These data highlight the potential of this platform to comprehensively characterize adapter UniCAR-T cells, determining their functional heterogeneity, and thereby advance the development of next-generation modular CAR-T cell therapies.

P0687

New Technologies for CAR-T Characterization and Potency Testing

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The development and optimization of CAR-T cell therapies require robust and accurate methods for characterization and potency testing. This poster presents novel technologies designed to enhance these processes, focusing on the application of NanoBiT™ technology, HiBiT assays, Lumit® immunoassays and various genomic tools **for lenti plasmid and virus and CAR-T molecular characterization.**

HiBiT™ Technology for Monitoring Target Cell Killing: This innovative approach allows for rapid and mechanism-of-action based CAR-T potency testing. The assay employs HiBiT-expressing target cells, which release HiBiT into the medium upon lysis of target cells by the effector CAR-T cells. HiBiT binds to LgBiT in the detection reagent, forming a functional luciferase that emits light. The simple workflow integrates the plating of HiBiT target cells, the addition of CAR-T cells, and the detection of luminescence, streamlining the characterization process. Extended assay incubation times reveal serial target cell killing, crucial for evaluating CAR-T cell efficacy over time. We have built a comprehensive panel of HiBiT target cell lines, including both endogenous and engineered antigen-expressing cells, supporting the testing of various CAR-T constructs. The use of CD19/CD20 knockout cells further allows for the evaluation of Tandem and dual CAR-T cells specificity.

Lumit® Immunoassays for Cytokine Detection and p24 Lenti Viral titer: Homogeneous Lumit® immunoassays are simple, fast, and sensitive, requiring no washing steps. We have developed a suite Lumit® immunoassays for the measurement of cytokine secretion by CAR-T cells as well as p24 for Lenti viral titer.

This suite of technologies offers a robust framework for the detailed characterization and potency testing of CAR-T cell therapies, aiming to enhance their development and clinical application.

P0688

Comparative development of second-generation CAR-Ts targeting CLL-1: analysis of CD28 and 4-1BB co-stimulatory domains in generated AML cell models

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Cancer immunotherapy, especially adoptive cell therapy (ACT) utilizing chimeric antigen receptor T cells (CAR-T), has significantly advanced the treatment of blood cancers. Nonetheless, it faces substantial obstacles in treating acute myeloid leukemia (AML), such as the absence of an

appropriate target antigen, complexities in CAR-T cells manufacture, and the impact of tumor microenvironment (TME). Thus, further studies are needed to achieve better treatment outcomes, especially those focused on the design of the CAR, and more specifically the co-stimulatory domains, which play an indispensable role in activation and persistence among other main characteristics.

To this end, we designed and compared the efficacy of anti-CLL-1-based CAR-T cells equipped with either 4-1BB or CD28 co-stimulatory domains. We established AML target cell line models, including Raji, K-562, MOLM-13, HL-60, THP-1, and U-937, and used GFP-NanoLuc for facilitating *in vitro* and *in vivo* tracking. We also used CRISPR-Cas9 technology to generate a negative control cell line lacking CLL-1 expression. Subsequently, we developed protocols to evaluate the *in vitro* efficacy and safety of the anti-CLL-1 CAR-T therapy. Our preliminary results demonstrated that transduction and sorting achieved 100% expression of GFP-NanoLuc in the obtained cell lines. Each cell line exhibited a characteristic expression profile of the analyzed markers, with several significant differences, particularly in CLL-1 expression, mimicking the high heterogeneity and variability observed in AML patients. We also successfully generated a U-937 GFP-NanoLuc CLL-1 *knock-out* (KO) cell line with CRISPR-Cas9 technology as specific negative control. Finally, through co-culturing CAR-T cells and U-937 cells, we demonstrated that our CAR constructs were capable of specifically lysing this CLL-1 expressing AML cell line.

In conclusion, we successfully achieved 100% GFP-NanoLuc expression in the Raji, K-562, MOLM-13, HL-60, THP-1, and U-937 cell lines, facilitating both *in vitro* and *in vivo* studies. Additionally, we used genomic editing techniques to create a U-937 CLL-1 *knock-out* cell line, which serves as an essential negative control in our experiments. These cell lines serve as excellent models for mimicking the varied levels of marker expression observed in AML patients, making them ideal for comprehensive studies. Furthermore, we have successfully designed and produced two anti-CLL-1 CAR-T constructs with 4-1BB and CD28 co-stimulatory domains, both demonstrating specific lytic capacity against CLL-1 expression in the U-937 cell line. Ongoing experiments aim to compare the *in vivo* efficacy of CAR-Ts anti-CLL-1 based on these different co-stimulatory domains.

P0689

Super resolution microscopy reveals gene-transfer strategy-induced disparity of CAR expression affecting CAR-T cell function in an antigen density dependent manner

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Adoptive immunotherapy with genetically engineered chimeric antigen receptor (CAR) T cells has proven to be a transformative treatment for advanced leukemias and lymphomas and is currently under intense investigation to translate this success to the treatment of solid tumors.

For the development of advanced CAR-T cell products with optimal safety and efficacy it is critical to determine, how distinct gene-transfer strategies affect spatiotemporal CAR expression and modulate T cell effector functions.

We therefore established a test platform enabling us to manufacture, characterize and compare CAR-T cells engineered by either, lentiviral transduction (LV), Sleeping Beauty (SB) transposon-based gene-transfer or CRISPR-Cas-mediated targeted CAR insertion (KI) into the TRAC locus, to express CAR constructs that cover a range of different target and epitope specificities and graded affinities. Further, the streamlined manufacturing process allows focus on gene-transfer related effects, and aims to minimize unrelated production derived phenotypic differences.

Building on this platform, we characterized the generated CAR-T cells phenotypically in detail by flow cytometry as well as transcriptomics, accompanied by determination of CAR copy number variation (CNV) via droplet digital (dd)PCR, and analysed CAR surface expression and organization by *d*STORM super-resolution microscopy.

Further, we employed a library of target cells with distinct antigen densities to functionally characterize the CAR-T cells regarding short vs. long-term cytolytic activity, cytokine secretion and proliferation capacity *in vitro*, as well as the rate of antigen-induced cell death (AICD).

We found significant characteristic variations in the number of GOI integrations that translated to pronounced differences in absolute number, spatial distribution and dynamics of CAR expression during sequential stimulation campaigns.

On the surface of LV/SB CAR-T cells, we detected a significantly higher CAR density compared to TRAC knock-in CAR-T cells and the observed range of CAR expression was narrower for TRAC KI CAR-T cells in both, CD4 and CD8 T cells.

In functional experiments, CAR-T cells showed a graded response (SB>LV>KI) in *in-vitro* cytokine-release assays in very-high antigen settings. Further, SB CAR-T cells showed advantage in short-term (< 8 hours) lysis assays across multiple effector-to-target (E:T) ratios in this setting. Both, SB and LV CAR-T cells, displayed superior short-term cytolytic activity compared to KI CAR-T cells in ultra-low antigen settings. With longer follow-up (> 20 hours) LV/SB CAR-T cells and KI CAR-T cells were equally effective independent of antigen settings. Intriguingly, we observed a significantly lesser extent of activation-induced cell death (AICD) in KI CAR-T cells, suggesting that lower CAR amount and density could provide protection from overstimulation. Distinct CAR expression levels engineered using the same gene-transfer method confirmed a major role of CAR expression responsible for the observed differences in functionality between gene-transfer methods. Further investigation in pre-clinical *in vivo* models is ongoing.

Taken together, these data show that non-targeted (LV/SB) and targeted (KI) CAR insertion result in distinct patterns of CAR expression and regulation that translate into distinct anti-tumour reactivity. These results suggest that matching the method of gene-transfer and CAR expression to target antigen levels in the respective tumour entity can provide critical advantages for CAR-T cell therapy.

P0690

Investigation of the influence of CAR T cell manufacturing procedure adjustment on the functionality and fitness of the drug product

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Chimeric antigen receptor (CAR) T cell therapy has demonstrated sustained clinical benefits in the treatment of haematological malignancies and is regarded as a promising approach to cancer treatment. An automated manufacturing process for CAR T cell Drug Products (DP) using the CliniMACS Prodigy® has been established and is subject to continuous review and improvement. Critical material parameters (CMPs), such as the supplementation of human AB (hAB) serum, may influence the quality of the DP. In this study, we describe our efforts to characterise the stability, functionality and mitochondrial state of the CAR T cell DP in relation to the duration of hAB serum supplementation for a fresh DP.

To investigate the influence of the duration of hAB serum supplementation, a series of experiments were conducted utilizing the CliniMACS Prodigy® instrument. The established 12-day manufacturing procedure was performed with hAB serum supplementation either for the first 5 days of culture or for the full 12 days. Control samples were taken throughout the process to monitor the expansion and transduction efficiency of the DP. Subsequently, the resulting DP was characterized for stability and functional activity over a 72-hour storage period. In addition, the levels of reactive oxygen species and the mitochondrial content within the different conditions were compared.

Prolonged supplementation with human AB serum for 12 days demonstrated a significant impact on the final DP. During the shelf-life of fresh DP, enhanced cellular fitness and extended viability of the cells were observed with prolonged hAB supplementation, suggesting that hAB serum is a critical material parameter influencing the CAR T cell quality. In the future, it may be beneficial to place greater emphasis on investigating the mitochondrial function of the DP to more fully characterise cellular fitness. In conclusion, these findings emphasize the importance of continuous process improvement and review of the critical material parameters.

P0691

Identification of drugs with a systems biology approach to counteract T cell exhaustion

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CD8+ T cells are an essential component of the immune system that efficiently recognize and kill virally infected- and cancer cells through the release of perforins, granzymes, and Fas ligands. However, in conditions like chronic infection or cancer, T cell activity is hampered by persistent stimulation, which causes a dysfunctional state better known as exhaustion. Exhaustion is a dynamic process in which CD8+ T cells become hypo-responsive to antigen stimulation and lose their immune surveillance capabilities to fight off pathogens and tumor cells. Functionally, exhaustion is characterized by low effector cytokine secretion (IFNγ, TNFα, IL-2), impaired cytotoxicity due to the accumulation of inhibitory immune checkpoint receptors (PD-1, TIM-3, LAG-3, etc) that upon engagement with their ligands in tumor cells, prevent the activation of T cell-mediated immunity. Moreover, poor proliferative capacity and persistence are other hallmarks of exhaustion. Finally, emerging evidence indicates that exhausted T cells exhibit a distinct epigenetic landscape, suggesting that epigenetic remodeling is directly involved in the

transcriptional control of T cell exhaustion. Indeed, T cell exhaustion limits the wide applicability and effectiveness of CAR-T therapy in solid tumors. The onset of exhaustion in engineered T cells by persistent CAR signaling remains a substantial barrier to overcome for successful immunotherapy.

It was recently proposed that small molecules to regulate cellular processes in a desired fashion can be identified by a computational tool named "*DECCODE*" (Drug Enhanced Cell Conversion using Differential Expression). DECCODE compares the expression profile of interest to those of a large collection of drug-treated cells, providing a similarity score for each comparison. Here we used DECCODE to identify drugs that counteract exhaustion and obtain more persistent T cell activity. DECCODE compared a collection of transcriptional profiles of drug-treated cells with transcriptional data of T cells at different stages of activation and exhaustion. We then selected drugs associated with an expression profile more closely aligned with activated T cells and divergent from exhausted ones. Among the candidates tested, we identified a histone-deacetylase inhibitor, that significantly downregulates the expression of immune checkpoint receptors and transcription factors normally overexpressed in exhausted T cells. We found that this drug also improves the functionality and cytotoxic effects of HER2 CAR-T cells against ovarian cancer cells (SKOV3) and CD19 CAR-T cells against acute lymphoblastic leukemia (OKT3). Furthermore, the drug has been proven to significantly enhance the stemness marker TCF7 on CD8+ T cells, inhibiting the effector differentiation.

Taken together, our results make us hypothesize that the drug may prevent epigenetic remodeling associated with T cell exhaustion. Future studies will focus on a better understanding of the drug's mechanism of action along with *in vivo* tests to assess the therapeutic potential.

P0692

Developing image-based cell assays to effectively monitor T-cell apoptosis and CAR expression

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Chimeric antigen receptor (CAR)-T cell therapy is one of the novel cellular therapeutic options for cancer patients, such as the B-cell malignancies. To effectively develop and manufacture CAR-T products, it is imperative to identify and assess the critical quality attributes (CQAs) of CAR-T cells. Recently, the Food and Drug Administration (FDA) has published the chemistry manufacturing and controls (CMC) guidance for human gene therapy. Cell concentration, cell viability, and CAR expression are important quality attributes that should be reliably measured during the entire process. In this work, we developed an image-based method to quickly count viable T cell, measure viability, and assess CAR expression. Using this new method, we compare different gene delivery procedures to make CAR T cells, mostly through the electroporation process. Our results showed that high percentage of apoptotic cells were introduced during the electroporation process, regardless of the CAR vectors. Cell viabilities were checked daily with AO/PI dual fluorescent staining method and the viability data were correlated with apoptosis data. On Day 1 after transduction, measured cell viabilities of transduced SupT1 cell samples decreased to ~60% - ~70%. Compared to measured cell viabilities of un-transduced and electroporation SupT1 controls of over 90%, it was identified that the introduction of plasmids, other than the electroporation process itself, induced 20 – 30% additional cell death. Our apoptosis data further confirmed that most of the cell death after electroporation were potentially due to cell apoptosis. The transduced SupT1 cell samples were able to recover as their

viabilities increased to >90% on Day 3. Percentages of CAR expression in transduced SupT1 cell samples were characterized by staining with APC-conjugated specific anti-CAR antibody. As for the percentages of CAR expression measurements, we observed similar CAR expression results in different transduced SupT1 cell samples on Day 1 and Day 2. We confirmed the percentages of CAR expression results using the flow cytometer and did not see significant differences between Cellaca® PLX Image Cytometer and flow cytometer results. With the advantages of ease of use, visual verification with captured cell images, and higher-throughput capability, Cellaca® PLX Image Cytometer may be potentially used as the convenient benchtop system for rapid assessment of the quantity and quality of CAR-T cells, which may ultimately improve the productivity of development and manufacture of CAR-T products.

P0694

Programmable, multiplex CAR T cell engineering with Genome Editing induced Gene Silencing (GEiGS®) technology

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Adoptive cell immunotherapy products, such as CAR T cells, have demonstrated clinical efficacy across many trials but despite these success, solid tumour indications remain challenging. Across the field, there are concerns that a lack of T cell persistence may leave patients vulnerable to relapse. Gene knockout approaches, often using CRISPR/Cas9 and targeting T cell checkpoints, have shown promise in mitigating T cell exhaustion and overcoming the inhibitory signals of the tumour microenvironment (TME). However, complete gene knockout may limit normal T cell signalling and memory subset formation, curtailing the durability of cells in vivo, and is unsuitable for conventional multiplex gene editing due to the genotoxicity of multiple DNA double strand breaks.

Laverock Therapeutics is leveraging Genome Editing induced Gene Silencing (GEiGS) technology to create the next generation of T cell therapies that can address these unmet clinical needs.

GEiGS technology retargets endogenous miRNAs to facilitate multiplexed programmable, stable and tunable gene silencing. By editing miRNAs that are differentially expressed in the anti-tumour response, this platform holds the promise to create adaptable CAR T cells that only alter their gene expression levels in the desired immune environment.

To establish a GEiGS T cell platform, we designed reengineered miRNAs targeting key T cell immunomodulatory factors using our proprietary computational pipeline. Candidates were screened in a high throughput pooled screening pipeline, to identify effective novel constructs. Once validated, HDR genome editing regents were designed, and incorporated into a CAR T cell manufacturing protocol. Edited cells were assessed for protein knockdown, phenotype and improved efficacy in in vitro killing- and cytokine secretion assays.

Our results to date show the GEiGS technology can be effectively deployed to engineer donor-derived CAR-T cells, and bring us closer to realising our vision of a next generation adoptive T cell products with improved efficacy, safety and accessibility, overcoming many of the limitations with existing approaches.

P0696

Comparison of Manual vs. Automated Extraction Techniques for RCL Clinical Monitoring

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Transduced cell therapies, for example CAR-T cells, offer a powerful therapeutic strategy for treatment of various cancers and auto-immune diseases. CAR-T therapies and analogues based on other immune cell types are most commonly transduced using lentiviral vectors to integrate the selected Chimeric Antigen Receptor gene construct into the selected cell line type. The use of lentiviral vectors introduces the risk of patient exposure to the same, with a particular concern regarding Replication Competent Lentivirus (RCL). While there is an expectation for cell-culture based RCL testing as part of the release of the cell product, there is also a necessity to establish the absence of RCL over the course of a clinical study within patients treated with using lentiviral-transduced cell therapy products. The current FDA recommendation for clinical monitoring of such patients is for testing of blood/PBMCs for the absence of RCL before dosing and, periodically over the course of the first for a year following dosing. If positive results are observed, clinical monitoring will continue as long-term follow up testing for up to 15 years. Often required as a primary safety endpoint, RCL clinical monitoring may need additional regulatory oversight (e.g. CLIA) on sample testing for assurance of patient safety.

One recommended analytical endpoint for such testing is the detection of a highly conserved sequence found in the lentiviral VSV-G Envelope Protein gene. VSV-G is essential for viral transduction of the CAR structure into the specific cell of interest. Frequently conducted in parallel, molecule-specific, Vector Copy Number (VCN) PK endpoints are also supported for which sensitivity is an important factor to assess cell therapy persistence over the course of the trial. Therefore, an off-the-shelf RCL qPCR method targeting VSV-G in blood samples has been validated at Labcorp Early Development Laboratories to support RCL testing as well as testing of VCN through extraction of DNA from a larger quantity of blood than might be used for other applications. This allows for the VCN and RCL endpoints to be assessed from the same primary sample and to maintain the sensitivity needed to assess cell therapy persistence over time.

The RCL qPCR method was originally validated alongside a 2 mL manual blood DNA extraction. However, an automated method has recently been validated alongside the RCL qPCR to offer a lower sample input requirement of 1 mL, ultimately reducing costs while remaining suitable for parallel VCN analysis. Here, we compare the consistency, yield, and analyte recovery efficiency of both the manual and automated extraction methods via the RCL qPCR assay for assessment of the most favourable method. Also, the implications to sample volume requirements and analytical expenses are discussed in more detail. The over-arching benefits of aligned VCN and RCL analysis endpoints in clinical sample analysis are considered in comparison.

P0697

Enhanced CD19-specific CAR T cells through editing of exhaustion-associated miRNAs

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Despite the success of chimeric antigen receptor (CAR) T cell-based immunotherapy, a sizable proportion of patients treated with CAR T cells experience disease relapse. This may be due to the low persistence or exhaustion of CAR T cells due to their continuous exposure to the antigen. Several genes have been identified as promoters or antagonists in pathways that control T cell exhaustion, such as those that encoding for inhibitory receptors, and their inhibition via blocking antibody or genome editing strategies has shown some promise in the clinics. However, the complexity of these pathways and the increasing number of genes involved, suggest that multiplexed alteration is necessary to eventually improve CAR T cells performance. Micro RNAs (miRNAs) have naturally evolved to simultaneously regulate multiple genes, thus impacting on several physiological pathways. In an effort to generate CAR T cells that resist T cell exhaustion, we sought to identify critical miRNAs that regulate this process and alter their expression via precise genome editing strategies to render CAR T cells resilient to tumor-induced exhaustion. First, we identified the optimal conditions for manufacturing large quantities of CD19-specific CAR T cells using lentiviral-mediated transgene delivery and identified conditions that consistently yielded over 50% of CAR-positive T cells. We then established an assay to induce CAR T cell exhaustion *in vitro* through repetitive antigen exposure. We exploited this system to identify miRNAs that either promote or antagonize exhaustion using transcriptome and miRNA sequence analyses of CAR T cells collected at different time points. We then applied the 'castling technology' to reduce the expression of exhaustion-promoting miRNA and simultaneously upregulate the expression of miRNA that antagonizes exhaustion. The gene editing pipeline for the production of 'castled' CD19 CAR T cells involves the swap of deleterious miRNA with the miRNA that antagonizes exhaustion by using two CRISPR-Cas9 ribonucleases together with an appropriate repair template supplied through an adeno-associated viral vector. The combination of lentiviral mediated CAR transgene expression with the genome editing procedure did not affect the viability of CAR T cells. This study opens novel opportunities towards the generation of improved allogeneic cancer immunotherapies and might potentially extend the therapeutic use of this platform to more resilient tumor types in the future.

P0698

Development & testing of lipid nanoparticles as in vivo vehicle for solid tumor gene therapy

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Despite a global increase in solid tumor-related disease burdens, limited treatment options exist for high-risk and relapse cases. Adoptive T-cell therapy, utilizing chimeric antigen receptor (CAR) T cells shows potential for addressing this issue, yet hurdles like poor T-cell infiltration and immunosuppressive tumor environments persist. To tackle these challenges, our research group is developing a personalized gene therapy. We modify patient tumor cells to express immune

modulators, altering the tumor environment for better CAR-T cell infiltration and enhanced destruction of tumor cells. This subproject aims to create a targeted lipid nanoparticle-based delivery system (tLNP) for in vivo CRISPR-based gene therapy. It packages and delivers Cas9 mRNA, tumor specific sgRNA, and double-stranded donor DNA templates simultaneously to tumor cells, facilitating tumor-specific CRISPR-mediated knock-in repair. This combination of immunotherapy and gene therapy conquers the challenge of reaching all tumor cells, a significant issue in solid tumor gene therapy. Analysis via dynamic light scattering (DLS) shows consistent and reproducible results using a commercially available kit and own formulations. To date we've successfully co-transferred these therapeutic agents in vitro using cationic lipids and explored new LNP formulations for better efficiency. With the commercial kit we achieved transfection rates of up to 100% for RNA and 50% for DNA in tumor cells and knock-in rates of up to 20%, validated through dPCR and flow cytometry. While those results were accompanied by dose-related toxicity, we saw DNA transfection rates of up to 75%, knock-in rates of up to 35% and no toxicity using our own formulations. In a new approach, we also use single-stranded donor DNA which makes the use of larger quantities of nucleic acid components possible, potentially leading to higher knock-in rates while avoiding dose-related toxicity in tumor cells. For in vivo treatment, promising candidates are planned to be tailored with tumor-specific antibodies for targeted gene therapy delivery. We could show that lipid nanoparticles are a highly effective delivery method for mRNA, sgRNA and DNA and therefor suitable for in vivo delivery of CRISPR-based gene therapy components, resulting in possible applications not only in antitumor therapy but also in gene therapies in many other areas.

P0699

Systematic *in vitro* assay enables functional screening of gene editing strategies to prevent exhaustion of CAR T cells

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Adoptive T cell therapy relying on the expression of a chimeric antigen receptor (CAR) has broadened the success of cancer immunotherapy. With the ability to target tumor cells by recognizing tumor-associated antigens, CAR T cells are among an ever-growing line of novel cell-based therapies to treat malignant diseases. Clinical reports of patients receiving CAR T cells targeting the B-cell receptor CD19 for the treatment of various types of leukemia and lymphoma have shown previously unprecedented levels of remission. Despite this encouraging success, the therapy failed in a substantial number of patients. Further investigations revealed the development of an “exhausted” T cell phenotype as one of the leading causes of failure. Exhaustion entails a dysfunctional state consequent to persistent stimulation and internalization of inhibitory signals. First evidences of this state arose from chronic viral infections and expanded to the physiopathology of tumors upon better understanding of the tumor microenvironment (TME). Given the complex and divergent nature of the TME, *in vitro* models that could recreate the conditions leading to an exhausted phenotype bear the potential to single out key partakers of the process and to devise molecular strategies to prevent exhaustion to generate improved CAR T cell products. Here, we describe an *in vitro* assay designed to drive CD19-specific CAR T cells to a state of exhaustion, verifiable by characteristic functional features and phenotype. We co-cultured a defined number of CD19-specific CAR T cells with a fixed amount of CD19-positive

NALM6 cells. As the co-culture progressed, we adjusted the number of target cells to keep the effector-to-target ratio constant. In this condition, we observed that CAR T cells derived from different healthy donors showed differences in the kinetics of expansion and the onset of exhaustion. Nonetheless, the impairment in cytotoxicity at the endpoint remained comparable across replicates and coincided with decreased stemness, reduced secretion of pro-inflammatory cytokines and increased expression of the exhaustion-related marker CD39. To demonstrate that this assay could be valuable to evaluate gene editing strategies that prevent exhaustion, we use the CRISPR/Cas9 system to inactivate the Ten-Eleven Translocation-2 (*TET2*) gene, a key epigenetic regulator associated with T cell exhaustion. Knocking out *TET2* caused a delayed expansion, improved cytotoxicity, increased stemness, and decreased CD39 expression in line with previous reports. These results indicate that the developed *in vitro* assay can be confidently exploited to explore novel gene editing interventions that prevent exhaustion and thus enable the development of improved CAR T cell products.

P0700

Tumour-derived G-CSF promotes immunosuppressive microenvironment in osteosarcoma model, reducing response to CAR.GD2 T-cells

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Sarcomas are rare mesenchymal tumours, representing about 10-15% of all childhood cancers. GD2 is an ideal target for chimeric antigen receptor (CAR) T-cell therapy due to its overexpression in various solid tumours. This study investigates the potential use of iCasp9.2A.GD2.CAR-CD28.4–1BBζ (CAR.GD2) T-cells for treating GD2-positive sarcomas and examines factors shaping the tumour microenvironment in these cases. GD2 expression on primary sarcoma biopsies revealed positivity in 54.7% of cases, with the highest percentages observed in osteosarcoma OS (range from 1.4% to 92.3%) and rhabdomyosarcoma RMS (range from 1% to 70%), and lower in Ewing sarcoma EWS (range from 1.0% to 3.42%). Among sarcoma cell lines, it was observed that OS (MG-63, 143B, U-2OS), embryonal RMS (RD), and alveolar RMS (RH4) cell lines exhibited high GD2 expression (%GD2+ ≥70%). *In vitro*, CAR.GD2 T-cells significantly eradicate GD2-positive sarcoma cells, including OS and RMS cells. Specifically, CAR.GD2 T-cells demonstrated significant tumour regression on 143B and RD cell lines in both two-dimensional (2D), even at a very low E:T ratio of 1:32 ($p=0.0044$ for 143B and $p=0.0130$ for RD cell line, respectively) and in three-dimensional (3D) culture models ($p\leq0.001$ for 143B and $p\leq0.001$ for RD cell lines) compared to NT T-cells. Additionally, they exhibited substantial cytokine production, which correlates with their killing activity. In *in vivo* xenograft models, CAR.GD2 T-cells significantly controlled tumour growth and improved survival in mice bearing RD RMS (tumour bioluminescence CAR.GD2 vs NT: 2.5E7 vs 5.7E9 p/s/cm^2/sr, $p\leq0.001$) and OS tumour (143B tumour bioluminescence CAR.GD2 vs NT: 5.0E6±4.7E6 p/s/cm^2/sr vs 1.6E10±1.5E10 p/s/cm^2/sr, $p=0.047$; U2-OS tumour bioluminescence CAR.GD2 vs NT: 2.5E6 vs 1.6E10 p/s/cm^2/sr vs 1.6E10±1.5E10 p/s/cm^2/sr, $p=0.047$). Notably, CAR.GD2 T-cells maintained stable expression

and effective antitumor activity over prolonged exposures, with shifts in memory profile indicating a transition from naive to central and effector memory states. In the RD xenograft mouse model, treatment with CAR.GD2 T-cells extended overall survival compared to controls (69.5 days, p=0.0413). Similarly, in an orthotopic 143B OS model, CAR.GD2 T-cells led to decreased tumour growth and enhanced survival (46 days, p=0.0052), with stable CAR expression and significant expansion of human T-cells in mouse peripheral blood. Moreover, a robust expansion of murine MDSCs was observed in the 143B osteosarcoma xenograft model, associated with high levels of G-CSF and CXCL8, indicating their role in immunosuppression. The presence of immunosuppressive MDSCs, particularly in the 143B model, was confirmed through increased myeloid cell populations in peripheral blood compared to that observed in controls (total count/50uL blood: 57775 vs 4375 p≤0.001), highlighting the challenge of the immunosuppressive microenvironment in maintaining long-term antitumor efficacy of CAR.GD2 T-cells. The presence of immunosuppressive murine MDSCs significantly reduces long-term anti-tumour effectiveness of infused CAR.GD2 T-cells. *In vitro* tumour derived G-CSF, but not CXCL8, was found to induce also a robust expansion of human MDSCs, supporting the immunosuppressive role of this chemokine.

In summary, CAR.GD2 T-cells show promising preclinical efficacy against GD2-positive sarcomas, with significant antitumor activity and persistence. However, the immunosuppressive TME, driven by MDSCs, poses a challenge, necessitating strategies to improve therapeutic outcomes.

P0701

Cytosine base editing facilitates selective HLA-silencing to generate broadly compatible allogeneic CAR T cells for off-the-shelf use

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Over the past decade, the landscape of blood cancer treatment has been transformed by Chimeric Antigen Receptor-T (CAR-T) cell therapy. Despite the remarkable efficacy of CAR-T cells in targeting B cell malignancies, hurdles such as complex manufacturing processes, high costs, and time-intensive expansion procedures persist within autologous T cell engineering. Addressing these challenges, allogeneic CAR-T cells have emerged as a promising alternative. However, rejection of allogeneic T cells due to human leucocyte antigen (HLA) disparity remains a key barrier to the realization of universal donor T cell therapy, complicated further by the high polymorphisms of HLA genes.

A potential solution involves the knockout of HLA class I by disrupting the *beta 2 microglobulin* (*B2M*) gene, creating universal donor cells. However, T cells lacking HLA class I expression may be recognized by natural killer (NK) cells through a missing-self mechanism, limiting their persistence. To mitigate this, combining HLA-matching with the knockout of mismatched HLA genes using CRISPR-Cas9 has been proposed. This approach ensures edited cells are protected from host T cell rejection, resistant to NK cell lysis, and capable of presenting a wide range of peptides for immune surveillance. However, conventional CRISPR-Cas-induced DNA double-strand breaks (DSB) pose a safety concern due to on-target aberrations and potential chromosomal rearrangements.

Here, we investigated whether DSB-free base editing could silence specific HLA alleles, enabling selective elimination of mismatched HLA on T cells. We designed 11 guide RNAs (gRNAs) to target at least 2 of the 10 most common HLA alleles, directing the cytosine base editor to introduce premature stop-codons. To ensure specificity and avoid unintended editing of the HLA-E gene locus or other genes, we developed a Python script that allows pre-selection of suitable gRNAs according to the donor's HLA repertoire.

Testing in cells from donors of known HLA type achieved knockout rates up to 90%, measured by flow cytometry. Some cross-reactive editing could be detected, as certain gRNAs designed for *HLA-A* or *HLA-B* induced high KO rates in *HLA-C* and vice-versa. We combined gRNAs effectively, leaving only one or two types of HLA-I molecules on the cell surface, potentially allowing a higher relative HLA-match between donor and host. Comparing susceptibility to NK cells, *B2M* knockout T cells lacking HLA class I were efficiently lysed, whereas base edited T cells retaining *HLA-A* or *HLA-C* expression were significantly protected from NK cell cytotoxicity. Preliminary results indicate base editor-mediated HLA-disruption can be combined with simultaneous CRISPR-Cas12a knock-in of a CD19-specific CAR into the CD3-zeta locus, achieving up to 39% CAR expression in HLA-engineered T cells. This insertion disrupts TCR expression, minimizing potential graft-versus-host reactivity.

Our method demonstrates the potential for using multiplexed base editing to create HLA-engineered allogeneic 'off-the-shelf' CAR-T cell banks with enhanced recipient compatibility and therapeutic potential, circumventing immune mediated rejection while ensuring safety.

P0702

Controlling glioblastoma with CAR T cells targeting an alternatively spliced domain of Tenascin C

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Glioblastoma (GBM) is a highly aggressive and incurable primary brain tumor, with limited therapeutic advances made over the past decades. The urgent need for developing new therapies to improve patient outcomes is thus clear. Chimeric antigen receptor (CAR) T cell therapy has shown breakthrough success in hematological cancers but has demonstrated limited efficacy in clinical studies for GBM, primarily due to tumor heterogeneity, antigen loss, and the lack of cancer-restricted antigens. Targeting the cancer-specific alternatively spliced domain D of the extracellular matrix protein Tenascin C (TNC) could help overcome these limitations. In GBM, the alternatively spliced TNC isoform is extensively expressed by cancer cells and adheres to the cell surface. Here, we developed a 2nd generation CAR using the publicly available R6N scFv clone with a CD28z costimulatory domain. TNC-CAR T cells successfully bound to soluble TNC and displayed an activation phenotype when cocultured with purified TNC protein, supernatants from TNC-expressing GBM patient-derived cells, and digested GBM patient tumor samples. Furthermore, TNC-CAR T cells efficiently killed naturally TNC-secreting patient-derived GBM cell lines and exhibited bystander cytotoxicity against TNC-negative cell lines when incubated with TNC-positive cells. We also demonstrated that soluble TNC makes TNC-negative cells susceptible to TNC-CAR T cells, highlighting the advantage of targeting a soluble molecule. In vivo, NSG mice bearing patient-derived GBM tumors treated with TNC-CAR T cells showed increased T cell infiltration and apoptosis, leading to improved survival. Altogether, these results suggest that targeting the tumor-specific TNC splice variant D with CAR T cells is a promising approach that

could address some key limitations in the treatment of glioblastoma. However, additional preclinical studies in immunocompetent models are needed to fully understand the impact of targeting TNC on the tumor microenvironment and to optimize the therapeutic potential of this strategy.

P0703

Targeting O-Acetylated GD2 with CAR T cells as a novel tumor-specific approach in neuroblastoma and brain tumors

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Implementation of chimeric antigen receptor (CAR) T cell therapy in solid tumours has been slowed down due to a highly immunosuppressive tumour microenvironment (TME), tumour heterogeneity and a lack of tumor-specific antigens. Targeting aberrant tumor glycosylation is an interesting strategy to circumvent the latter. Looking into developing safe CAR T cell therapies, we generated CAR T cells targeting the O-Acetylated derivative of GD2 (OAcGD2). OAcGD2 is expressed in tumors of neuroectodermal origin, and shows minimal expression in healthy tissue. We developed different OAcGD2-targeting CAR constructs and showed that a longer CAR molecule enhances antigen recognition. We showed that anti-OAcGD2 CAR T cells display cytotoxicity and effector cytokine production when incubated with neuroblastoma, pediatric brain tumor and glioblastoma cell lines in vitro, supporting further development. Next steps include assessing safety and efficacy *in vivo* inimmunodeficient murine models as well as toxicity and changes induced to the TME in immunocompetent models. If successful, this work could lead to the implementation of a clinical trial testing anti-OAcGD2-CAR T cells, possibly in combination with CAR T cells targeting additional glioblastoma antigens.

P0704

Generating off-the-shelf TCR libraries for treatment of viral infections by CRISPR/Cas9-mediated TCR engineering

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Reactivation of latent viral infections poses a significant threat to immunocompromised individuals, such as those who have undergone hematopoietic stem cell or solid organ transplantation. Traditional antiviral medications are often insufficient due to limited efficacy and the emergence of drug resistance. Adoptive T-cell transfer has shown promise, particularly in

addressing viruses like cytomegalovirus (CMV), Epstein-Barr virus (EBV), and adenovirus (AdV). However, despite the success of using virus-specific T cells from seropositive donors in clinical studies, the logistical and technical challenges of isolating these cells from suitable donors limit the broad applicability of this therapy.

To address this, we engineer T cells with virus-specific T-cell receptors (TCRs) through CRISPR-Cas9-mediated techniques. Our method, termed 'orthotopic TCR replacement' (OTR), facilitates the generation of near-physiological engineered T cells with consistent and predictable *in vivo* behaviours.

We are currently generating “off-the-shelf” libraries of virus-specific TCRs that can serve virtually any patient. To this end, we have developed a pipeline that enables the rapid identification and selection of candidate TCRs for therapy. We first identified the minimum number of Human Leukocyte Antigen (HLA) molecules needed to cover 95% of the global population. Using this HLA framework, we searched for immunodominant epitopes from various viral antigens. These peptides were used to first screen over 100 donors for reactive T cells, which were then isolated based on activation markers. Through high-throughput single-cell sequencing (scRNAseq), we uncovered both donors with heterogeneous TCR repertoires and those dominated by a few super-expanded clones. We also identified varying levels of cytokine expression independent of clonal expansion. By re-expressing selected TCRs and performing retrospective transcriptomic analysis, we linked their viral reactivity to defined gene signatures. Applying a genetic scoring system to new scRNAseq data sets, we can predict the reactivity of newly identified TCR sequences on a high level, thus adding to the diverse array of TCRs which we have accumulated, which cover various HLAs.

The ultimate goal is to advance OTR-engineered virus-specific T cells into clinical applications. By doing so, this project not only addresses the immediate clinical need but also paves the way for future advancements in T-cell engineering and adoptive immunotherapy.

P0705

CancerPAM: A multiomics-based pipeline for identifying genomic targets for CRISPR knock-in for cancer gene therapy

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Solid tumors represent a significant therapeutic challenge, necessitating new approaches for target identification. Multi-omics analyses enable the discovery of novel targets for innovative gene therapies, such as CRISPR/Cas9-mediated transgene knock-in, offering promising new treatment approaches. Unlike CRISPR/Cas9-mediated gene knock-out through indel formation, HDR-dependent knock-in can integrate transgenes either in-frame (non-disruptive) or with exogenous promoters in non-coding regions or non-essential genes.

Here, we present CancerPAM, an automated multi-omics pipeline for CRISPR/Cas9 knock-in target identification that enhances feasibility and reproducibility. This command-line tool, written in Python and coordinated via Snakemake, will be made available on GitHub upon publication. By analyzing both the cancer genome and the genome of healthy tissue from patients, new tumor-specific PAM sites can be discovered, providing safer and more effective CRISPR targets. In various datasets, including a cohort of 54 neuroblastoma patients, nearly 10,000 potential CRISPR

targets were identified, accounting for almost 8% of all called variants. Patients with more variants in the cancer genome have a higher likelihood of discovering new PAMs with favorable properties for knock-ins.

The pipeline integrates computational analysis for target prediction and ranking, ensuring precise and efficient identification of the most promising genomic targets for personalized in vivo gene therapy. An established ranking algorithm compares the new tumor-specific PAMs within a sample or patient. To determine a score, potential targets were sorted according to various features and weighted accordingly. Relevant features include copy number, gene expression levels at the variant site, and various scores evaluating CRISPR efficiency and specificity. Avoiding off-target effects is particularly crucial for in vivo applications. Additionally, the pipeline features an interactive platform where users can analyze their datasets in detail, ensuring transparent and straightforward result interpretation.

The tool's results were compared with a manual multi-step selection process used to identify the most promising knock-in targets in two neuroblastoma cell lines. These targets were validated through in vitro knock-in experiments, and the pipeline successfully identified and ranked these targets based on their knock-in performance.

One potential use is a personalized gene therapy approach we are developing to address challenges in CAR T cell therapy. This CRISPR knock-in-based therapy modifies neuroblastoma cells in vivo, enabling them to express transgenes encoding T cell-attracting and stimulating chemokines and cytokines. This strategy aims to reshape the tumor microenvironment favorably and promote the activation and infiltration of CAR T cells. While our approach is being demonstrated in neuroblastoma for immunotherapy improvement, it is also applicable to other tumor entities and transgenes. CancerPAM will play a crucial role during therapy development by finding ideal targets in individual patients. Moreover, while it is currently not used for detection of potential CRISPR knock-out targets, CancerPAM could easily be repurposed to detect novel PAM sites in essential genes for knock-out-based antitumor therapy.

In conclusion, CancerPAM represents a significant advancement in the identification of tumor-specific CRISPR/Cas9 targets, facilitating the development of personalized gene therapies and potentially improving treatment outcomes for various solid tumors.

P0706

Improving the whole-cell cancer vaccine efficiency through immunogenic cell death

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Cancer recurrence after primary treatment is not uncommon, making subsequent treatments more challenging and impacting mortality rates. Currently, there are no standard and effective preventative treatments against recurrent cancer or metastasis, underscoring the importance of developing effective preventative therapies. Whole-cell cancer vaccines, produced using a patient's own cancer cells, can contain a variety of tumor antigens. However, due to their autologous origin, overcoming immune tolerance has been a long-standing research challenge. Immunogenic cell death (ICD) induction may be a promising approach. ICD can be triggered by

cellular stress resulting in release of specific damage-associated molecular patterns (DAMPs) or stress proteins on cell surface or extracellularly. These immune activation cascades, coupled with presence of tumor antigens, may successfully breakthrough immune tolerance and activate an effective anti-tumor immune response. In this study, we explored how to effectively induce ICD in cancer cells for application in whole-cell vaccines. Heat treatment and radiation are common physical methods for cellular inactivation. Here, we inactivated murine MC38 (colon adenocarcinoma), 4T1 (mammary carcinoma), and Panc02 (pancreatic ductal adenocarcinoma) cancer cells by heat and/or radiation treatments. We hypothesize that heat and radiation combination treatment can inactivate cancer cells more efficiently, exacerbate release of stress proteins to enhance anti-tumor immune response and break through immune tolerance. Our results showed that while different cancer cells had different tolerances to singular heat or radiation treatments, heat and radiation treatment (HRT) combination inactivated cancer cells more effectively. HRT increased the expression of heat-induced heat shock protein 70 kDa (HSP70) and radiation-induced high mobility group box 1 (HMGB1) protein, which are both DAMP molecules that can stimulate immune response activation. When HRT-treated cancer cells were used as whole-cell vaccine in a tumor challenge model *in vivo*, it elicited a stronger anti-tumor immune stimulation and tumor suppression than whole-cell vaccines treated with heat or radiation alone. *In vitro*, we found that HRT cancer cells co-cultured with antigen-presenting cells (APCs) were not only more easily phagocytosed by APCs, compared to singular treatment-treated cancer cells, but also induced higher CD86 activation in these APCs. Additionally, we found that when HRT-treated whole cell vaccine was injected by a needleless jet injector instead of the conventional needle-syringe method, the pressurized ejection caused further cellular degradation of whole-cell vaccine contents to release more DAMP molecules. This method enhanced vaccine-mediated immune stimulation and anti-tumor effect. In conclusion, we have demonstrated that using dual heat/radiation inactivation treatments when processing whole-cell vaccines can efficiently inactivate cancer cells and improve overall vaccine anti-tumor effects. Whole-cell vaccine can also be further enhanced when combined with an appropriate administration method. Therefore, whole-cell vaccine efficiency can be greatly improved and may potentially become a more viable option for recurrent cancer therapy.

P0707

Boosted CAR T cell for improved safety and efficacy against low antigen density antigen ALK

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CAR T cell therapy has proven highly successful in haematological disease in recent years, especially CD19 targeting CARs have resulted in complete remission in various leukaemias. In the solid tumour context however, limitations such as on-target off-tumour toxicity are prominent. Identification of a suitable target antigen with expression limited to tumour tissue is therefore crucial. Anaplastic lymphoma kinase (ALK) is expressed in multiple paediatric and adult cancers, including neuroblastoma, retinoblastomas, rhabdomyosarcomas and non-small cell lung cancer, whilst being absent from normal tissue, making it an ideal target for CAR T cell therapy. However, expression levels on tumour cells are below the threshold required to appropriately initiate a sustained activation response by standard CD8h/tm CD28z CAR T cells. We generated a novel anti-ALK binder (ALK8), which in CAR-T format has higher sensitivity to low antigen density and made further modifications to boost the CAR. Firstly, to reduce the antigen threshold required for

a sustained response, a second generation ALK8 CAR was modified to enhance signalling through a CD28 hinge/transmembrane (h/tm), previously shown to improve CAR function and addition of a second zeta ITAM to boost signalling upon antigen encounter. In vitro co-cultures with low ALK expressing neuroblastoma cell lines showed improvements in cytotoxicity, proliferation and cytokine production. Secondly, CAR response to low antigen density was augmented by co-expression of a chimeric costimulatory receptor (CCR) to an antigen co-expressed on the target cell at high levels, namely B7H3. A dual CAR conferring a cytotoxic response to ALK, boosted by a B7H3 specific CCR in different heterodimerising and non-heterodimerising configurations of the h/tm were tested. We hypothesised that matched h/tm (either CD8a or CD28 in both CAR and CCR) would lead to cytotoxicity against B7H3 in the absence of ALK by signal transfer to the CAR through dimerization, however only observed evidence of this in a 28h/tm combination. To reduce the likelihood of any off target effects mismatched h/tm domains in CAR and CCR were taken forward to retain specificity of the ALK CAR but increase proliferation and cytokine production through the B7H3 CCR. Combination of the ALK8 binder with a boosted CAR design leads to augmentation of a proliferation and cytokine release signal whilst retaining specificity, defining an ALK-CAR capable of sensing naturally occurring low antigen density of ALK, allowing specific targeting of a multitude of solid cancers whilst limiting the likelihood of toxicity to healthy tissue.

P0708

Enhancing CAR-T Therapy Through Engineered EVs Targeting AML-Specific Antigens

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CAR-T therapy has emerged as a breakthrough in the treatment of hematological malignancies, offering a personalized and targeted approach to cancer treatment. Strong clinical outcomes in B-cell leukemias and lymphomas underscore the potential of CAR-T cells to achieve significant remission rates. However, translating this therapy to other malignancies such as acute myeloid leukemia (AML) presents substantial challenges, including the absence of a specific tumor antigen and the impact of the tumor microenvironment (TME). Recent research has explored extracellular vesicles (EVs), including exosomes and microvesicles, as innovative carriers of bioactive molecules. These EVs can be engineered to target specific cells within the TME, thereby modulating the immune response and enhancing CAR-T cell functionality.

In this study, we propose combining EVs engineered to target a frequently upregulated surface antigen in AML with a CAR-T therapy targeting CLL-1, an overexpressed marker in AML blasts and leukemic stem cells (LSCs). These EVs will deliver specific silencing RNA (siRNAs) to mediate local immunosuppression in the TME. Preliminary findings indicate that targeted EVs effectively home to their intended cells, and ongoing studies are evaluating the impact of these targeted EVs on reducing immunosuppressive molecules, improving CAR-T cell proliferation, and enhancing persistence.

This targeted immunomodulation strategy holds promise in overcoming current limitations of CAR-T therapy and advancing more effective and durable treatments for hematological malignancies. Integrating these approaches provides a robust framework for studying exosome interactions with target cells and supports their potential applications in innovative therapies, including CAR-T cell therapy.

P0709

Cost-effective CAR-NK cell manufacturing using NK Production Kits

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Introduction

Although gene-modified NK cells with chimeric antigen receptor (CAR) have been shown to enhance the effector cell function and antigene-specificity against several tumor targets, the usage of CAR-NK cell-based therapy is still faced with several obstacles, including low efficiency of CAR-transduction and limited cell expansion. Hillgene has developed the series of cell production kits containing positive controls supporting on cell therapy development. These kits can broadly meet customers' needs in the whole product development cycles from the early stages of R&D through IND stages until the clinical stages.

Key Technology and Method

CAR-Nk Cell Production kits have been developed to mimic the manufacturing process of NK cell isolation, activiation, transduction, expansion and formulation & cryopreservation. Lentiviral vectors supplied in the kit are manufactured using Hillgene platform technology and process and are compliant with regulations. Each CAR-specific kit contains selected reagents, starting materials and the protocol, providing the defined culture conditions and well-established process. Following the protocol, customers can experience a successful cell production. These kits can also be used as controls for trouble-shooting activites.

Conclusion

The kits designed for increasing the technically successful rate and accelerating the pace on engineered NK cell therapy development and commercialisation. (1) The kits help on reducing the risk and cost of early R&D production. In the early R&D process, the higher uncertainty, the higher risk and cost. Based on the large cell and vector libraries built, the kits can help clients effectively select suitable cells or vectors and optimize them, ultimately reducing trial and error costs and increasing the success rate of R&D. (2) The kits can shorten the R&D time. Due to the high diversity and complexity nature of viral vectors, large-scale manufacture of viral vector is not only time-consuming and costly, but also has certain bottlenecks in verification at the cell level. The cell production kit containing defined starting materials and well-established processes can ensure the success of cell production even for the first-time players. Moreover, the kits can help customers in handling some hard-to-work cell types, i.e. NK cells and accumulate practical knowledge in cell expansion. (3) Cells produced using the kits meet the regulatory compliance requirements for product quality and safety. The kits are designed for fulfilling regulatory and GMP-ready requirement.

P0710

HLA-G+ induced T-Regulatory Cells (iG-Tregs) for GvHD Prevention: From Pre-Clinical Evaluation to First-in-human Phase I/II Clinical Trial

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Graft-versus-host disease (GvHD) is a life-threatening complication following allogeneic hematopoietic cell transplantation (allo-HCT). In order to fight GvHD, we sought to express in T-cells the immunomodulatory molecule HLA-G which is crucial in shielding the "semi-allogeneic" fetus from maternal immune rejection, but it is epigenetically silenced post-birth. We have previously demonstrated that hypomethylating agents like Decitabine, induce stable expression of HLA-G in T-cells, converting them into immunosuppressive T-regulatory cells (Tregs). Here we provide preclinical data on the efficacy of HLA-G+Tregs (iG-Tregs) in GvHD prevention and preliminary findings from the first-in-human phase I/II clinical trial with iG-Tregs (EUDRACT 2021-006367-26). iG-Tregs were generated after a 3-day exposure of anti-CD3/CD28-activated T-cells to Decitabine and assessed in vitro and in vivo in a mouse GvHD model. Compared to the untreated control cells, iG-Tregs showed a significant increase in CD3+HLA-G+cells [Δm 16% (5.86-28%) vs 1.7% (0.12-3.9%), p<0.0001] and displayed identifying traits of T-reg populations (LAG-3/TIM-3). Functionally, iG-Tregs exhibited reduced in vitro alloreactivity against allogeneic targets over control cells (1.8% vs 49% lysis, p<0.0001) while in vivo, 67% of iG-Treg-treated mice survived until sacrifice (d84, p=0.0019), in contrast to control mice which succumbed to clinically and histologically confirmed GvHD. iG-Tregs in vitro, also reduced the alloreactivity of T-conventional cells (Tconv) (p=0.04), while in vivo, their co-infusion with Tconv cells delayed or prevented acute GvHD, significantly improving their survival over Tconv-treated mice (p=0.005). Importantly also, iG-Tregs maintained the graft-versus-leukemia (GvL) effect in a myelogenous leukemia mouse model, where iG-Tregs+K562cells-treated mice survived without GvHD or leukemia until the day of sacrifice unlike those receiving K562-only or Tconv+K562 which developed leukemia or GvHD, respectively and succumbed early post-infusion (p=0.0057). Good Manufacturing Practice (GMP) validation runs confirmed the feasibility of producing clinical scale/grade iG-Tregs with induced HLA-G+ expression [Δm 18% (15-31.9%)] and lack of alloreactivity (7% over 28.3% of Tconv, p<0.0001). Decitabine treatment resulted into a T cell receptor repertoire's renewal, as only a small fraction (6,06%) of identical "public" clonotypes were identified in the pre- and post-Decitabine samples, thus inducing a polyclonal and highly diverse iG-Treg population. Likewise, a distinct transcriptomic signature of iG-Treg products was evident by the differential expression of genes post- over pre- treatment, involved in pathways related to T-cell mediated immune response, activation/proliferation and signaling. We subsequently preceded with initiation of a phase I/II dose escalation (0.1/0.5/1.5x10^6 iG-Tregs cells/kg) study in an allo-HCT, HLA-matched sibling donor setting, anticipating early tappering

and discontinuation of cyclosporin in patients infused with donor iG-Tregs. Thus far, three patients with high-risk leukemias received iG-Tregs enriched in HLA-G+CD3+ cells 13.61% (Δm). As of last follow-up (up to d311), two patients remain disease- and GvHD-free, while one patient experienced skin grade-I GvHD that resolved with topical treatment. Immunophenotypic monitoring revealed a rise in HLA-G+ T-cells at week 1 followed by an increase in natural Tregs at week 8. Overall, our pre-clinical and preliminary clinical results demonstrate the feasibility of generating iG-Tregs and their promising efficacy as a novel immunotherapy strategy against GvHD.

P0711

Preclinical modelling and mechanistic insights into CAR-T cell induced neurotoxicity

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Immune effector cell-associated neurotoxicity syndrome (ICANS) occurs frequently in cancer patients undergoing chimeric antigen receptor (CAR)-T cell therapy. Its severity is expected to increase with the release of next-generation CAR-T cell products; however its pathophysiology is still poorly understood. Current hypotheses mainly come from autopsies performed on brains of patients who suffered from fatal neurotoxicity and preclinical *in vivo* and *in vitro* models able to recapitulate the human disease are limited. Recently, syngeneic mouse models of CAR-related neurotoxicity have been described; unfortunately, they did not allow to study ICANS that develops upon interaction of human CAR-T cells with human immune and tumor cells.
Here we exploited a hematopoietic stem and progenitor cells (HSPCs) humanized mouse model able to recapitulate CAR-related toxicities after treatment with human CAR-T cells. The model requires infusion of immunodeficient mice with human cord-blood CD34+ cells, luciferase+ NALM-6 cells and peripheral blood-derived CD19 CAR-T cells. It has shown its ability to recapitulate CAR-related cytokine release syndrome (CRS) thanks to the presence of human myeloid cells, which are primarily responsible for the systemic release of cytokines causing the syndrome. Leveraging the model to gain a deeper understanding of ICANS pathophysiology, we observed the development of multifocal brain haemorrhages in about the 45% of mice treated with human CD19 CAR-T cells. Neurotoxic manifestations affect mainly the cerebellum, both in terms of extension and incidence. By magnetic resonance imaging we observed the onset of hypointense lesions in deep brain structures and identified contrast agent extravasation events, suggesting an increased permeability of the blood-brain-barrier (BBB) that is typical of patients

with ICANS. Accordingly, increased concentrations of the Evans blue dye were detected in the brains of treated animals. Behavioural tests revealed changes in locomotor abilities, likely providing a clinical relevance to the phenomena occurring in deep brain areas. Mice with acute neurotoxicity were those experiencing more severe weight loss, reduced survival, and increased serum levels of proinflammatory cytokines, suggesting that systemic inflammatory response driven by CAR T cell activation-induced CRS could foster neurotoxic manifestations. Of note, serum samples from mice experiencing acute neurotoxicity significantly impair BBB components, as observed by challenging a fully human model of BBB, including brain endothelial cells, pericytes, and astrocytes derived from human induced pluripotent stem cells. Specifically, we detected a decrease in the integrity of endothelial cells, as indicated by reduced transepithelial electrical resistance and mRNA expression of zonula occludens-1 (ZO-1), a tight junction protein. Additionally, astrocytes exhibited increased reactivity, evidenced by elevated mRNA expression of intermediate filaments as glial fibrillary acidic protein (GFAP) and vimentin (VIM). These findings suggest that the humanized mouse model and the *in vitro* BBB model are promisingly able to recapitulate ICANS-related complications, providing us with tools for the identification of actionable targets and prediction of neurotoxic potential of CAR-T cell products.

P0712

A novel strategy for off-the-shelf T cell therapies evading T cell and NK cell rejection

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Autologous chimeric antigen receptor (CAR) T cell therapies have shown great success for treating several types of hematological malignancies, but their widespread use has been hindered by several challenges, including manufacturing failures and the high cost of individualized products. This has sparked a strong desire for "off-the-shelf" allogeneic products. A key safety concern with allogeneic T cells, the risk of graft-versus-host disease (GvHD), has largely been addressed by removing the endogenous T cell receptor (TCR) using gene editing technologies. However, key challenges remain for generating persistent allogeneic T cells due to the recognition of mismatched human leukocyte antigen (HLA) alleles by host T cells or the recognition of cells lacking HLA class I (e.g., when removed via beta-2-microglobulin (*B2M)* knockout) by NK cells, both of which can lead to rejection of such allogeneic T cells by the host immune system. Other approaches involving deep or prolonged lymphodepletion of the host can improve allogeneic T cell persistence but come with an increased risk of infections. To address these challenges, we developed an allogeneic T cell strategy by leveraging a dual CRISPR/Cas9 cleavase/base editor and lipid nanoparticle (LNP) delivery platforms to perform one site-specific insertion for a targeting CAR or TCR and four knockouts, removing the endogenous TCR (via *TRAC*) to prevent GvHD, and disrupting *HLA-A*, *HLA-B*, and the class II transactivator (*CIITA*) to prevent T and NK cell rejection. Edited donor T cells did not induce GvHD in an immunocompromised mouse model over a 90-day evaluation period. Cells knocked out for *HLA-A*, *HLA-B*, and *CIITA* were protected from rejection by alloreactive T cells and NK cells when the residual HLA-C allele was matched to the hosts. The matching approach is further simplified using donors homozygous for *HLA-C*, achieving HLA matching across a broad patient population with a minimal number of donors. Using our proprietary T cell engineering process, we successfully generated allogeneic T cells incorporating four gene knockouts plus site-specific insertion of a tumor-specific TCR or CAR, with high yields and efficiency. Importantly, these allogeneic T cells had comparable functional activity to the control cells engineered without the *HLA-A*, *-B*, and *CIITA* edits in *in vitro* and *in*

vivo assays, suggesting no unfavorable functional impact from these gene edits. In summary, we have successfully developed a differentiated "off-the-shelf" approach for CAR-T and TCR-T therapies, which is expected to be cost-effective to produce and durable without requiring intense lymphodepletion. This promising strategy is being applied to our cell therapy pipeline, and its implementation in other cell types is being investigated.

P0713

Novel SdAb-based CAR-T cells against CD33 present improved efficacy for the treatment of AML

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Acute myeloid leukaemia (AML) is the most frequent acute leukaemia in adults characterized by a significant genomic heterogeneity. Despite recent advances with the approval of over 7 new therapies, prognosis in these patients particularly in the relapse and refractory setting is poor, with median overall survival bellow 8 months. Thus, there is an urgent need for new therapeutic strategies. The current generation of CAR-T cells has shown spectacular efficacy in certain hematological cancers. However mature clinical data with CAR-T therapies in AML have yet to be published and only a small number of pilot studies report the use of CAR-T cells against CD33. Single-domain antibodies (SdAb) present interesting features for the development of innovative CAR-T cell designs including improved antitumor efficacy, since recognition of several epitopes reduces tumor escape, and reduced immunogenicity that would prevent the immune response against the CAR-T.

The main aim of this work was to identify and characterize novel SdAb against CD33 to improve the efficacy of CAR-T therapies in AML. Novel SdAb against CD33 were generated by building a library from immunized llamas. Phage display enrichment was performed to identify specific SdAbs, that were subsequently sequenced and subcloned in bacterial expression vectors for purification. SdAbs were characterized in terms of affinity, binding kinetics, and epitope competition by ELISA, surface plasmon resonance (SPR) and biolayer interferometry (BLI). SdAbs-based CARs were generated replacing the scFv sequence of a 2nd generation 4-1BB construct. Activation profile and tonic signal were evaluated in Jurkat triple reporter system (TPR). SdAb-based CAR-T cells were produced by lentiviral infection of activated T cells from healthy donors. Cytotoxicity was evaluated by using Bright-Glo™ Luciferase Assay System. *In vivo* antitumoral efficacy was evaluated using xenograft AML models in NSG mice.

After phage display SdAbs specific for CD33, corresponding to different families were identified. SdAbs (one per family) were selected for further characterization, and 3 CAR constructs were selected based on their affinity, binding kinetics, the absence of tonic signalling as well as their specific activation in response to $CD33^+$ cells. Selected constructs were used to generate CAR-T cells, that were evaluated *in vitro* and *in vivo*, using an scFv-based CAR-T as

control. All CAR-T cells presented high cytotoxic activity and produced high levels of cytokines (IFNg, IL2 and TNFa) in response to AML cell lines expressing different levels of CD33 (MOLM13, MV4-11 and HL60), without statistical differences. However, all SdAb-based CAR-T cells showed improved antitumoral efficacy *in vivo* compared to control CAR-T cells. Interestingly, we observed that SdAb1- and SdAb16-CAR-T cells, with moderate or high affinity, displayed better antitumoral response than SdAb3-CAR-T cells, with lower affinity.

Overall, our results allowed us to identify and characterize several SdAb-based CAR-T cells with different affinities, that outperformed the antitumoral efficacy of scFv-based CAR-T cells that are currently under clinical evaluation. These results indicate that these novel SdAb-based CAR-T cells against CD33 could be a promising therapeutic option for AML.

P0714

Synthetic T-rEx: A synthetic biology approach to overcome T cell exhaustion

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Synthetic biology aims at reprogramming cell fate and functions by genetic circuits designed to sense and respond to cues in the intracellular and/or extracellular environment. These *smart interfaces* process endogenous information and implement robust responses enabling high specificity and self-containment of desired output activation. Synthetic biology is emerging as potential game changer in engineered cell-based immunotherapies, by using safety switches and linking specific input sensing to gene expression induction. Adoptive T cell therapy, the engineering of a patient's T cells with tumor-targeting receptors as therapeutic agents, has revolutionized the treatment of blood tumors; however, despite the remarkable success there are issues that still need to be addressed to expand its broader applicability. It was recently demonstrated that **T cell exhaustion**, which occurs in natural immune response, is detrimental to the antitumor capacity of CAR-T cells when they are chronically exposed to the antigen. Current immunotherapies to reinvigorate exhausted T cells target immune checkpoint blockade with anti-CTLA-4 and/or PD1 monoclonal antibodies. Despite the clinical benefits, there are still two main drawbacks: 1) the **efficacy and long-term** effect of this approach which depends on several factors, including the stage of the dysfunction and 2) **adverse effects** relative to high dosage, and toxic metabolites. We developed Synthetic T-rEX, a synthetic circuit platform that aims at engineering $CD8^+$ T cells resistant to exhaustion. Synthetic T-rEX includes a sensing module and an actuator module. The sensors are newly engineered synthetic promoters (SP) that respond to transcription factors (TF), whose expression **progressively increases from activation to exhaustion**. The actuators are classified in: 1) **TME immunostimulators** that include IL12p70, CCL21, antiPDL1 nanobody and 2) **T cell reprogrammers**, with newly engineered synthetic microRNAs targeting exhaustion associated genes. In this talk we specifically focus on the sensors: we designed and tested a library of 80 SPs. As point-in-case NR4A-SPs were further tested in primary T cells. We set up an *in vitro* exhaustion platform that induce a dysfunctional state in as short as 8 days, validated with phenotypical, functional analysis and RNA-sequencing. $CD8^+$ T cells were co-transduced with NR4A sensors and anti-HER2 CAR, and co-cultured with SKOV3 ovarian cancer cells, showing specific and reversible activation of the SPs: if cancer cells are removed **the circuits switches OFF**. By Time Lapse microscopy we show a different kinetics of activation for the variants of NR4A SPs, indicating a wide choice available to control timing and strength of therapeutic release. Finally, by coupling the sensors to therapeutic molecules we observe the controlled and reversible release of cytokines when T cells progress towards the

exhaustion state. In summary, we developed a versatile strategy to overcome T cell exhaustion that operates locally in the TME and induces a genetic switch from ON to OFF according to the external stimuli. This strategy holds potential for the development of more effective and persistent cell-based immunotherapy in solid tumors.

P0715

Toxin-free replacement of beta-thalassemia hematopoietic stem cells reverses disease phenotype

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Current untargeted cytotoxic conditioning regimens for hematopoietic stem cell transplantation (HSCT) have been directly or indirectly associated with transplant related morbidity and mortality limiting HSCT's wider use for non-malignant diseases. Recent pre-clinical and early clinical data indicate that alternative approaches such as monoclonal antibody (mAb)-based immunotherapies targeting the receptor tyrosine kinase c-KIT (CD117) expressed on the surface of human hematopoietic stem and progenitor cells (HSPCs) are generally safe. Blocking stem cell factor (SCF) binding to CD117 prevents proliferation resulting in in vivo HSPCs depletion. However, mAb-based conditioning necessitates a washout phase of the depleting antibody before engraftment of donor HSPCs and post-transplant redosing is impossible.

Engineered donor HSPCs shielded from a CD117-targeted immunotherapy could overcome these limitations. To this aim, we combined a novel, highly potent HSPC-depleting anti-CD117 mAb (CIM058) with CD117 variants carrying single amino acid substitutions that reduce CIM058 binding by more than 1000-fold. All selected variants exhibit an SCF binding affinity in the range of wildtype (wt) CD117. TF-1 cells expressing CD117 variants were insensitive to blocking mAb but retained SCF-dependent growth with EC50 values comparable to wt TF-1 cells. Furthermore, SCF-induced phosphorylation of CD117 Tyr719 was dose-dependent in TF-1 cells expressing CD117 variants even in the presence of a blocking mAb, indicating that SCF binding and signalling is intact. We next engineered the most favorable CD117 variant into mobilized peripheral blood HSPCs using a CRISPR/Cas9 HDR approach. HSPCs expressing the CD117 variant displayed normal hematopoietic differentiation potential in colony forming assays in vitro. Furthermore, they engrafted in immunodeficient NBSGW mice and contributed to multilineage hematopoiesis comparable to non-edited HSPCs. CIM058 administration to mice previously transplanted with variant engineered HSPCs resulted in the depletion of non-edited cells, while edited CD117+ cells were enriched. Secondary transplantation experiments confirmed successful editing of long-term HSCs.

Next, we assessed whether CD117 shielded HSPCs paired with CIM058 are beneficial in a genetic blood disease model. beta-thalassemia is a blood disorder caused by mutations in the b-globin gene. A humanized beta-thalassemia model was generated by engrafting immunodeficient NBSGW mice with mobilized CD34+ cells from a beta-thalassemia patient (IVSI-110/IVSI-110) successfully recapitulating disease phenotypes (including splenomegaly, increased ROS levels, reduced clonogenic capacity and impaired erythropoiesis). CIM058 injections in these humanized mice, enabled engraftment of CD117-edited HSPCs from a healthy donor. CIM058 treatment post transplantation, enriched for the edited, healthy cells and partially reversed b-thalassemia disease manifestations such as splenomegaly and impaired BFU-E formation and hemoglobinization, as compared to mice not receiving CIM058 treatment before or after transplantation. In order to further improve precision of CD117 variant engineering, we used prime editing which resulted in superior HSPC viability compared to HDR and no indel generation.

Taken together, our data demonstrate the feasibility of selectively depleting HSPCs expressing wt CD117 in a humanized model of beta-thalassemia while sparing engineered HSPCs. CIM058 treatment post transplantation enriched for CD117-shielded HSPCs, leading to partial rescue of the disease phenotype. We propose that this approach could allow toxin-free conditioning and post-transplant in vivo selection of healthy HSPCs.

P0716

Non-viral CRISPR knock-in strategies for endogenously regulated antigen receptors: insights from CAR, HIT/STAR, and eTRuC T cells

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Genetic engineering has revolutionised T cell therapy with rationally designed antigen receptors, exemplified by the success of chimeric antigen receptor (CAR) T cells. Traditional viral CAR delivery, however, presents several challenges: safety concerns due to secondary CAR^+ T cell lymphomas as a result of insertional mutagenesis, functional issues from strong artificial expression cassettes leading to exhaustion, and high costs and delays associated with GMP-grade virus production. Recently, we introduced a fully non-viral, CRISPR-facilitated knock-in strategy for highly efficient CAR reprogramming of T and NK cells by targeting truncated CAR transgenes into the *CD3ζ* gene. This in-frame integration generates functional CAR-*CD3ζ* fusion genes, leveraging the endogenous *CD3ζ* gene as the CAR activation domain, with expression regulated by the endogenous promoter and UTR regions. *CD3ζ* -edited CD19-CAR T cells demonstrated superior leukemia control in vivo compared to lentiviral delivery and TRAC integration.

Building on this success, we developed efficient knock-in strategies for two other classes of designed antigen receptors with T cell receptor (TCR) architectures: HLA-independent TCR/synthetic TCR and antigen receptor (HIT/STAR) and *CD3ε*-TCR-fusion construct (eTRuC). Likewise, these strategies target TCR-complex genes to create fusion genes composed of transgenic and endogenous parts, utilising the *TRAC* and *CD3ε* genes for HIT/STAR and eTRuC expression, respectively. We aimed to perform unbiased comparisons of CAR, HIT/STAR, and eTRuC architectures in primary human T cells using endogenously regulated expression systems. Initially, we tested the feasibility and functionality of these knock-ins with a standard CD19-

specific, FMC63-derived antigen-binding domain both *in vitro* and *in vivo*. All three receptors effectively reduced leukemia burden, although the response duration was shorter for TCR-based receptors (HIT/STAR, eTRuC).

Recent reports suggest that different receptor architectures significantly impact T cell activation thresholds, effector functions, and proliferation, with TCR-based architectures (HIT/STAR, eTRuC) demonstrating lower activation thresholds in viral overexpression systems. Activation thresholds are critical: while low thresholds can enhance therapeutic efficacy by preventing antigen escape, they also pose a risk of on-target off-tumour toxicity. This is particularly important for solid tumour antigens, which are often not exclusively expressed in tumours but also present in normal tissues at lower densities. To fully leverage the potential of novel antigen receptor classes, it is crucial to comprehensively characterise the interdependence of receptor affinity and antigen density for each receptor class. We conducted in-depth *in vitro* characterisation using solid tumour associated antigen-binding domains with varying affinities and target cells engineered to express different antigen levels. Consistent with previous findings, high-affinity receptors were activated by physiological antigen levels in advanced models with differences observed between the receptor classes. These findings underscore the importance of receptor architecture and affinity in designing safe and effective T cell therapies for cancer.

P0717

Automated, point-of-care manufacturing of FOLR1-directed CAR T cells for the treatment of ovarian cancer

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Treatment options for ovarian cancer patients are limited, and a high unmet clinical need remains for targeted and long-lasting, efficient drugs. Adoptive cell transfer therapies have made tremendous progress in the past years. Genetically engineered T cells expressing a chimeric antigen receptor (CAR) are promising drugs that can be directed towards a defined target and have shown efficient, as well as persisting, anti-tumor responses in different indications. Recently, we have reported the preclinical evaluation of a novel FOLR1-targeting CAR directed against ovarian cancer and potentially other FOLR1-expressing tumors.

Although T cell therapy development is advancing swiftly, producing a functional and robust cell product in the necessary quantities remains challenging. Currently available processes for the preparation of CAR T are laborious and include numerous manual handling steps, which increase the risk of errors and may impose safety risks. Consequently, there is a need to further develop reliable and automated manufacturing processes to produce high-quality cellular products for immunotherapy.

Therefore, this study aimed to develop an automated, closed, and good manufacturing practice (GMP)-compliant process for FOLR1-specific CAR T cells.

By using the CliniMACS Prodigy® Platform we could establish an automated and good manufacturing practice compliant process in a closed system, which meets the increasing regulatory requirements for cell-based therapeutics and additionally reduce hands-on time. We

optimized culture conditions to reduce the process time to 7 days. Starting from a cryopreserved leukapheresis we achieved a clinically relevant yield of FOLR1-specific CAR-engineered T cells. The cellular product mainly consisted of highly viable CAR-expressing T cells with an early memory phenotype. FOLR1-specific CAR T cells manufactured with the optimized process showed specific killing of ovarian cancer cells *in vitro* and in an ovarian cancer xenograft model *in vivo*. Moreover, the automated CliniMACS Prodigy Platform enables point-of-care (POC) manufacturing.

In conclusion, we established a robust, rapid process with high yields on a platform which is well suited for automated, and decentralized POC manufacturing of anti-FOLR1 CAR T cells and is well established for manufacturing of other CAR T cell products.

P0719

Gene-modified T cells targeting B7-H3 for the treatment of medulloblastoma: the race of two co-stimulus 4.1BB vs OX40

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Medulloblastoma (MB) is the most prevalent malignant brain tumor in children, accounting for 20% of pediatric brain tumors, with the highest incidence occurring between ages 5-7. Despite the availability of several aggressive therapies, 30% of patients succumb to the disease. Our aim is to bridge the gap between basic science and clinical application to develop more effective, less toxic therapies, improving patients' outcomes. Chimeric antigen receptor (CAR) T-cell therapy, has shown outstanding results in treating several hematological tumors. CAR T-cell therapy involves designing a specific CAR construct to arm cytolytic T-cells with a receptor that can recognize a tumor cell surface protein. A significant limitation of CAR T-cell therapy for solid tumors, including MB, is the limited number of target antigens with differential expression between tumor and normal tissues. One promising target identified for MB treatment is B7-H3. This antigen is involved in tumor cell proliferation, migration, invasion, and cancer stemness. It is expressed at low levels in most normal tissues but is overexpressed in various cancers, including MB.

B7-H3 expression was evaluated on primary tumor biopsies of 34 pediatric MB patients by flow-cytometry. In *in vitro*, as well as in *in vivo* models, B7-H3+ MB cells were targeted by two different third-generation CAR T-cells constructs, based on a novel anti-B7-H3 single chain variable fragment, incorporating either the CD28.4-1BB or CD28.OX40 costimulatory domains and the suicide gene inducible-caspase-9. B7-H3 was expressed in 65% of MB tumors. B7-H3 positivity was seen in 42.8% of WNT patients, 75% of SHH patients, 60% of G3 patients and 78.6% of G4 patients. In pre-clinical models, B7-H3.CAR.CD28.OX40z T-cells and B7-H3.CAR.CD28.4-1BBz T-cells exhibit comparable transduction level (72.46%±7.38% and 82.46%±6.69%), proliferation rate and *in vitro* cytotoxicity effect against several MB cell lines. In 6-days long-term co-culture, both B7-H3-CAR T-cells show a significant tumor control against D283 Med (G3/G4): the

residual tumor was 0.07%±0.09% for B7-H3.CAR.4-1BBz, 0.025%±0.050% for B7-H3.CAR.OX40z respect to un-transduced (NT) T-cells (86.80%±6.4%, p<0.0001); for DAOY (SHH) cell line the residual tumor was 0.025%±0.050 for B7-H3.CAR.4-1BBz, 0.31%±0.59% for B7-H3.CAR.OX40z) respect to NT T-cells (69.8%±19.7%, p<0.0001), and for MED 411-FHTC (G3) cells, the residual tumor was 0.22%±0.12 for B7-H3.CAR.4-1BBz and 0.35%±0.34% for B7-H3.CAR.OX40z, respect to NT T-cells (61.2%±19.2%, p<0.0001).

Despite the similar behavior *in vitro*, we observed different results *in vivo*. In orthotopic mouse brain tumor model, NSG mice were engrafted with patient derived xenograft MED-411FHTC. In this setting B7-H3.CAR.CD28.OX40z showed a marked tumor eradication effect at day +44 ($1.8x10^5$ p/sec/cm^2/sr and p <0.0001) respect to B7-H3.CAR.CD28.4-1BBz T-cells ($6.47x10^8$ p/sec/cm^2/sr and p=0.0003) and control NT T-cells ($2.74x10^9$ p/sec/cm^2/sr). The highest activity of B7-H3.CAR.CD28.OX40z T-cells was also demonstrated in an *in vivo* experiment using D284 Med tumor cell line with low B7-H3 MFI. In this model, we confirmed not only the anti-tumor effect of B7-H3.CAR.CD28.OX40 T-cells but also the absence of tumor relapse until day +100 post treatment. Based on these results B7-H3.CAR.CD28.OX40z construct is the most promising candidate for developing a clinical trial targeting MB in children and young adult patients.

P0720

T cell engineering using V(D)J recombination allows tumor growth inhibition in mice

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Autologous T cell therapies have shown clinical success in hematological malignancies, but they rely on prolonged and expensive ex vivo manipulations. Often applied as a late line of treatment, the efficacy of autologous therapies for both liquid and solid tumours is hampered by early T cell exhaustion, promoted by the prolonged culturing required for the scarcely retrieved cells. Conversely, “Off the shelf” allogeneic therapies reduce timelines and costs and may have favorable phenotypes, but they require disruption of the endogenous T cell receptor (TCR) to prevent graft vs. host disease (GVHD). We and others have demonstrated that nuclease mediated TCR disruption is associated with high rates of chromosomal aberrations.

Here, we propose “VDJ targeting”: a novel immunotherapy approach targeting AAV-delivered immune genes into the genome using V(D)J recombination in developing T cells. In particular, we use adeno-associated viral (AAV) vectors coding for a transgenic TCR/CAR gene followed by a recognition signal sequence (RSS) for the recombination activating gene (RAG) complex, which is active only during T cell development. VDJ targeting is thus nuclease-free and produces potent naïve T cells that, compared to the products of conventional engineering, may be transplanted in lower numbers and have increased persistence in both autologous and allogeneic therapies. In addition, Targeted T cells express only the desired receptors, due to allelic exclusion.

We have demonstrated VDJ targeting in ex vivo differentiated T cells of both human and mouse. Hematopoietic stem and progenitor cells (HSPCs) from mouse bone marrow were grown on OP9DL1 feeder cells in IL-7 and FLT-3 containing medium for 5 days, and then transduced

with 1E6 vector genomes per cell of an AAV-DJ vector coding for a CAR gene against CD19. 13 days following transduction up to 40% of the CD3+ cells were found to express the CAR, but only when an RSS was coded on the CAR vector. Co-culture of the CAR-T cells with syngeneic CD19 expressing Eμ-ALL cancer cells led to high levels of IFNγ secretion. Concordantly, ultrasound-guided intrathymic injections of AAV8 vectors in immunocompetent mice allowed for in vivo CAR gene integration by VDJ targeting. Similarly, human cord blood derived HSPCs were grown on the feeder cells with an SPEM-2 medium for 5 days and then transduced with an 7E5 vector genomes per cell of an AAV6 vector coding for either a CAR anti CD19 or a TCR targeting an NY-ESO1 peptide presented on HLA2-a2. 13 days post transduction, the receptor genes were detected on up to 36% and 12% of the CD3+ cells for the CAR and TCR respectively, and only when an RSS was coded on the vector. Co-culture with respective target cells allowed high levels of IFNγ secretion. Human T cells, engineered by VDJ recombination to express the TCR, were adoptively transferred into immunodeficient mice bearing the A375 melanoma cell line expressing NY-ESO1. Importantly, we demonstrate tumor growth inhibition and prolonged survival .VDJ targeting is thus an efficient T cell engineering technology applicable in the autologous, allogeneic and in vivo settings.

P0721

Remodeling of the Immune Microenvironment influences CD19 CAR T Cell Activity in B Cell Acute Lymphoblastic Leukemia

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In patients with B-cell Acute Lymphoblastic Leukemia (B-ALL), Chimeric Antigen Receptor (CAR) T cells targeting CD19 have achieved durable responses. However, the contribution of the tumor microenvironment on CAR T-cell fate and endogenous immunity remains incompletely understood. We hypothesized that BM immunological niche reacts to CAR T cell-mediated inflammation by modulation of immune response through the activation of inhibitory pathways and molecules. To verify this hypothesis we performed a transcriptional signature of CAR T cells and ME with the final aim to study the impact of TME on CAR T and the shape of immune response and define pathways of immune evasion in order to target mechanisms of resistance. Our group performed an academic phase I/II study (NCT03389035) with donor-derived anti-CD19 CAR T cells generated with Sleeping Beauty transposon in patients with B-ALL relapsed after allogeneic hematopoietic stem cell transplantation. We performed single-cell RNA sequencing and spectral flow cytometry on bone marrow (BM) resident immune cells of patients undergoing CAR T-cell infusion. To elucidate the mediators involved in the resolution of CAR T-cell-mediated inflammation, the single-cell transcriptome of patients' BM cells at early time points

post-CAR T-cell infusion was compared to pre-treatment samples at the moment of disease relapse and the infusion products. Eighteen sequencing libraries were analyzed, 6 libraries per patient, for a total of 71407 high-quality cells. Unbiased clustering of BM cells and infusion products was performed by UMAP embedding. Extensive integration of the dataset coming from the individual patients was observed. The more representative clusters were classified into infusion product, CD4 and CD8 endogenous population, B cells, myeloid cells, pDC, NK, and NK-T cells. We observed profound changes in the composition of BM after CAR T cells infusion compared to pre-treatment samples. Complete disappearance of the B cells associated cluster was observed after treatment. CAR T-cell treatment generated a remodeling of the BM microenvironment, with an increase in the myeloid cells (specifically monocytes), NK, NK-T, and exhausted CD8+ T cell populations. After CAR T-cell infusion, myeloid cells displayed a higher resemblance to myeloid-derived suppressor cells (MDSCs). GeneSet Enrichment Analysis (GSEA) showed significant enrichment in pathways associated with immunosuppression both in the myeloid compartment and in endogenous T cells and in CAR T cells. Of note, the genes involved are also strictly correlated to the generation of terminally exhausted T cells and the emergence of MDSCs. Spectral flow cytometry-based experiments validated our observation of an increase of monocytes, MDSC-like cells, NK, NK-T, and CD8 terminal cells. Modeling intercellular communication using NicheNet suggested the induction of immunosuppressive genes in myeloid cells by both CAR T cells and endogenous T cells. Using the same tool, we observed that myeloid cells induced significant signalling in both CAR T cells and endogenous T cells.We have characterized changes in BM TME after CAR T infusion. CAR T cells-mediated myeloid activation is associated with pathways of immune dysregulation that may dampen CAR T cell expansion and antagonize the effects of the therapy.

P0722

Development of CAR-T Cell Immunotherapy Against Head and Neck Squamous Cell Carcinoma for Patients with Fanconi Anemia

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Fanconi anemia (FA) is an autosomal inherited disorder caused by mutations in any of the 23 genes involved in the FA/BRCA DNA repair pathway. Clinically, FA is associated with bone marrow failure and cancer predisposition. FA patients are characterized by a 700-fold increased incidence of head and neck squamous cell carcinoma (HNSCC), which further increases after allogeneic hematopoietic stem cell transplantation (HSCT). The treatment of HNSCCs in FA patients is challenging due to their sensitivity to radio-chemotherapy, indicating the urgent need to develop new non-genotoxic anti-HNSCC therapies. Immunotherapy approaches based on

CAR-T cells should constitute a safe strategy for eradicating these tumors in FA patients. In addition, FA cells show several alterations, including oxidative stress imbalance, mitochondrial dysfunction, hypersensitivity to inflammatory cytokines and up-regulation of NKG2D ligands, all of which could impair the production and the functionality of CAR-T cells generated from FA patients.

In this work we aimed at the generation of CAR-T cells against FA-HNSCCs using lentiviral vectors (LVs) encoding for a second generation 4-1BB containing CAR that carries a pan-ErbB ligand (T1E) which recognizes eight out of nine ErbB family homo- and heterodimers. As previously described in primary HNSCCs from the general population, all tested FA HNSCC cell lines expressed high levels of ErbB members, particularly ErbB1 and ErbB2, confirming that these molecules should constitute good targets for the treatment of FA HNSCCs. For the generation of anti-ErbB CAR-T cells, peripheral blood T cells from healthy donors (HD) and FA patients were pre-stimulated, transduced with the anti-ErbB-CAR LV and expanded for up to 14 days. We evaluated the production of anti-ErbB CAR-T cells generated from FA patients in comparison with those generated from HDs, and evaluated their cytotoxic activity against different HNSCC cell lines, including *FANCA* mutated and *FANCC* mutated cell lines (VU-1365 and VU-1131, respectively), both of which expressed the EGFP and luciferase marker genes.

Despite the functional defects of FA cells, our data showed that CAR-T cells from FA patients could be generated as efficiently as those derived from HDs. The transduction efficacy observed in T-cells from HD and FA patients was similar, and also a comparable expansion rate, viability and percentage of CAR-T cells was observed in the final product derived from HD and FA patients. Analyses of the activation and exhaustion markers and of the different CAR-T cell subpopulations generated at the end of the manufacturing process was also analogous between HDs and FA patients. Similar to HD CAR-T cells, FA CAR-T cells mediated a marked cytotoxicity against FA HNSCCs, as deduced from analyses performed at four different effector:target cell ratios and different time points (60- 100% of tumor lysis). Additionally, preliminary *in vivo* results conducted in NSG mice carrying FA-HNSCCs have shown the efficacy associated to the intra-tumoral administration of anti-ErbB CAR-T cells against these tumors.

Taken together, our preclinical studies suggest that the generation of either autologous or donor-derived anti-ErbB CAR-T cells should constitute a safe non-genotoxic immunotherapy for either untransplanted or transplanted FA patients with HNSCCs.

P0723

Tolerogenic dendritic cell-based immunotherapy to prevent unwanted immune response in enzyme replacement therapy for lysosomal storage disorders

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Lysosomal storage disorders (LSDs) are caused by the lack of lysosomal enzymes. When available, enzyme replacement therapy (ERT) is the standard treatment for many LSDs, although immune

responses towards the infused enzyme can limit its therapeutic efficacy. Tolerogenic dendritic cells (tolDC) are pivotal in promoting antigen(Ag)-specific tolerance. We developed a protocol to generate tolDC by transducing monocytes with lentiviral-vectors (LV) encoding for IL-10 and a specific Ag fused to the invariant chain (Ii), which ensures stable presentation encoded Ag both in the context of MHC class I and in the context of MHC class II, as an exogenous Ag. This strategy generates tolDC endowed with the ability to present the encoded Ag to both CD4+ and CD8+ T cells in the presence of IL-10 ($DC^{IL\text{-}10/Ag}$). We previously showed the ability of $DC^{IL\text{-}10/Ag}$ in modulating Ag-specific T and B cell responses. With the aim of developing tolDC as enzyme-specific adjuvant immunotherapy for LSD patients undergoing ERT, we dissected the enzyme-specific immune response and the overall immune status of ERT-treated LSD patients (Mucopolysaccharidosis-IVA, Pompe disease and alpha-mannosidosis) and generated tolDC from patients' monocytes. While peripheral T cell response to the enzymes was barely detectable in all patients tested, we found variable titers of anti-drug antibodies (IgG) in patients' plasma samples, which could impact on Mannose-6-Phosphate-receptor- (M6PR-) mediated enzyme uptake in *in vitro* assays. Furthermore, we we observed increased levels of cytokines (TNFa, IL-18) and chemokines (MCP1, CXCL-13, MIP1a, EOTAXIN) in the plasma of LSD patients, compared to healthy controls (HC). In line with this, the phenotypic characterization of peripheral blood cells by flow cytometry, showed an upregulation of activation markers (HLA-DR) on monocytes and $CD8^+$ T cells and down-regulation of inhibitory markers (PD1) on T cells. Moreover, the frequency of granulocytes, in particular neutrophils, and antibody-producing B cells was higher in LSD patients compared to HC. Despite the activated phenotype, patient-derived $CD14^+$ cells differentiated in functional tolDC that expressed the tolerogenic marker HLA-G, modulated allogenic $CD4^+$ T cell responses, and promoted Tr1 cells as efficiently as those from healthy subjects.

To test the ability of $DC^{IL\text{-}10/Ag}$ to modulate adaptive responses to ERT we took advantage of the Mucopolysaccharidosis-I (MPSI) mouse model. We developed an ERT-like protocol based on weekly injections of a-L-iduronidase (IDUA) in which IDUA-specific $CD4^+$ T cell responses can be detected early after ERT initiation and anti-IDUA antibodies are induced after 4 weeks of treatment. In vitro testing demonstrated that engineered DC expressing an immunogenic IDUA epitope ($IDUA_{60\text{-}120}$) and IL-10 ($DC^{IL\text{-}10/IDUA}$) were able to dampen the Ag-specific $CD4^+$ T cell response. Furthermore, repetitive injections of $DC^{IL\text{-}10/IDUA}$ reduced circulating anti-IDUA antibodies, compared to control mice injected mice with control DC.

Our data indicate that, immune response to ERT occurs in treated patients and treated subjects display a peripheral pro-inflammatory signature. Despite this, monocytes from ERT-treated patients can differentiate into functional tolDC in vitro. Preliminary *in vivo* data suggest that tolDC immunotherapy effectively limits the immune response elicited by ERT, thus potentially fostering its efficacy.

P0724

A Functional CAR-T Cell Atlas to Unravel Regulatory Mechanisms of CAR-T Cells

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Over the last decade, adoptive cell therapy strategies with CAR-T cells have emerged as promising treatments for cancer, demonstrating remarkable success in certain hematological tumors. However, these therapies still present toxicities and face several limitations that compromise their long-term efficacy. While advances in single-cell sequencing have allowed remarkable progress in understanding CAR-T cells functionality, the underpinning regulatory mechanisms are still largely unknown. Importantly, there is still a lack of studies that leverage the vast amounts of public CAR-T cell data to dissect the molecular mechanisms governing the antitumoral response of CAR-T therapies.

In this work, we have developed the first CAR-T cell functional atlas from large public single-cell datasets, deepening our understanding of CAR-T cell transcriptional regulation. Specifically, we have collected around 1 million cells coming from 102 hematological patients and 14 healthy donors from 13 public CAR-T scRNA-seq studies, yielding an atlas of 414.000 $CD3^+$ CAR^+high-quality cells.

First, we validated key CAR-T features, such as 1) memory signatures of infusion products (IPs) associated with complete responses, 2) reduced cell type diversity enriched in activated $CD4^+$ memory in IPs from elder patients (>60 years old), or 3) the presence of classical monocyte-like CAR-T cells (IACs) in products from patients developing ICANS. Interestingly, we also detected unnoticed IACs at post-infusion samples and associated their presence with therapy-derived toxicities such as grade 3-4 ICANS. Besides, we described specific trajectories (from IP to year 9 after infusion) of cytotoxic $CD8^+$ CAR-T cells from complete responder patients, and deciphered key markers, like *PTPRM*, involved in long-term response. Finally, the atlas enabled us to delve into the scarcely reported association between gender, age, and therapy response. Importantly, the atlas will be broadly available to the biomedical community through scVI-hub. We envision this tool becoming essential for understanding CAR-T cells function, leading to improved therapies.

P0725

Dual targeting of PD-L1 and ErbB2 by CAR-NK cells enables specific elimination of solid tumor cells and overcomes immune escape via antigen loss

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Retargeting natural killer (NK) cells with chimeric antigen receptors (CARs) can be a powerful approach to overcome NK cell resistance of tumor cells. However, for some tumors, targeting a single tumor-associated antigen may be insufficient to trigger effective NK cell activation or may lead to selection of antigen-loss variants and tumor immune escape.

To overcome this hurdle, here we generated CAR-NK cells carrying two CARs targeting the tumor-associated antigens PD-L1 and ErbB2 (HER2), respectively. NK-92 cells were transduced with lentiviral CAR constructs, and their cytotoxicity against cancer cell lines of various solid tumor origins was compared to that of parental NK-92 and corresponding single target CAR variants.

Dual targeting significantly increased *in vitro* cytotoxicity against PD-L1 and ErbB2 double-positive tumor cell lines including breast, ovarian, pancreatic, lung and gastric cancer cells compared to single-target CAR variants. These results were also confirmed in 3D spheroid tumor models and *in vivo* xenografts. No off-target cytotoxicity was observed. At the molecular level, this enhanced cell killing can be explained by the synergistic activation of PLCγ and MAPK pathways. Incubation of cancer cells with IFN-γ further enhanced killing efficacy by upregulating PD-L1 expression. Furthermore, blocking experiments revealed that dual PD-L1/ErbB2-CAR NK-92 cells can overcome immune escape based on the loss or inaccessibility of a single target antigen.

In conclusion, we have shown that dual targeting of PD-L1 and ErbB2 enhances the efficacy of CAR-NK cells against otherwise difficult to treat tumors and counteracts potential resistance and immune escape mechanisms of cancer cells.

P0728

A systems biology approach to the identification of drugs able to counteract T-cell loss of functionality due to T-cell exhaustion in an in vitro exhaustion model

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CD8+ T cells can recognize and kill cancer cells by direct cytotoxicity (Perforins, GranzymeB, FasL). In a tumor context, in which antigens exposure is prolonged, CD8+ T cells progressively lose their functionality (IFNg, TNFa, IL-2) and cytotoxicity (PFN, GMZB) due to the accumulation of immune checkpoint receptors (PD-1, TIM-3, LAG-3, etc) in a physiological phenomenon called T-cell exhaustion. T-cell exhaustion is driven by key transcription factors (i.e., TOX, NR4A2) that tend to accumulate during persistent, chronic stimulation. These TFs ultimately lead to the accumulation of immune checkpoint (IC) receptor proteins and the epigenetic reorganization at the origin of T-cells loss of functionality (e.g. methylation of IL-2 promoter). To date, immune checkpoint inhibitors (anti CTLA-4, PD-1) are used to mitigate T cell exhaustion, but these do not guarantee long-term persistence and can trigger adverse effects after injection. Therefore, innovative approaches are highly needed.

Here we propose the combination of systems biology and immunology to identify drugs that can prevent exhaustion during persistent antigen stimulation. To this end, we applied the Mode of Action by NeTwoRk Analysis (MANTRA) to publicly available RNA-seq data (Singer M et al; Cell; 2016) to identify drugs whose associated expression profiles upon treatment are most similar and most divergent to those of activated and exhausted T-cells respectively (Carrella D et al; Bioinformatics; 2014). MANTRA is a computational tool for the analysis of the Mode of Action (MoA) of novel drugs and the identification of known and approved candidates for “drug repositioning”.

Two different drugs arising from MANTRA analysis (drug X, an agonist of TRPV1 ion channels; and drug Y, an antagonist of DRD2 dopamine receptors) showed to reduce key immune checkpoint receptors expression and to increase T-cells cytotoxicity against tumor cells after eight days of continuous CD3/CD28 stimulation in vitro. Strikingly, both drugs showed significant downmodulation of the NR4 transcription factors (NR4A1, NR4A2 and NR4A3), which are known early activated TF with a central role in T cell dysfunction.

RNA-seq data of CD8+ T cells treated at different stages of activation and exhaustion demonstrates that both drug treatments dramatically down-modulate ENPP1, the main negative feedback loop of cGAS-STING cytosolic DNA sensing pathway which, in turn, enhances IFN type I and type II responses. In addition, both drugs showed to up-regulate chemokine receptors, suggesting an enhanced migratory potential for CD8+ T cells treated with both drugs. Drug X also dramatically reduced the expression of VEGFR1 (FLT1) on treated CD8+ T cells and induced M1 polarization in THP-1 macrophages in vitro, whereas drug Y treatment on CD8+ T cells reduced the expression of exhaustion-related TFs SOX4 and NFATC4, while increasing the expression of IL-7 and up-regulating the main proteins involved in the unfolded protein response (UPR) (i.e. HSPA5).

Taken together, our results unveil drugs able to enhance cGAS-STING signaling and sustain type I and type II IFN signaling in a chronic stimulation setting as promising agents to counteract the hypofunctionality and hyporresponsiveness associated to T-cell exhaustion.

P0729

Targeting receptor tyrosine kinase ROR2 with CAR T cells is effective against hematologic and solid tumors, and well tolerated in mice

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The remarkable results obtained from autologous cell therapy of selected hematological malignancies using chimeric antigen receptor (CAR)-modified T cells has ignited a desire to expand this treatment approach to other diseases, including autoimmunity and solid cancers. However, CAR-T cell products have repeatedly failed to clear benchmarks of clinical efficacy in solid cancers. The development of CAR-T cell products targeting cross-entity tumor antigens

expressed in both hematological and solid cancer represent a unique opportunity to study the underlying mechanisms in more detail and to engage in an iterative optimization approach to augment CAR-T cell performance.

Here, we propose the receptor tyrosine kinase-like orphan receptor 2 (ROR2) as a novel target for CAR-T cell therapy with cross entity application in two representative hematological and solid cancers - multiple myeloma (MM) and clear cell renal cell carcinoma (ccRCC), respectively.

We studied the expression of ROR2 protein on the cell surface of MM cell lines and primary using flow cytometry and found ROR2 to be expressed in >90% of patient samples. Co-cultivation with a ROR2-specific CAR-T cell product (ROR2-CART) induced specific lysis of ROR2-positive tumor cell lines and primary samples, but failed to recognize ROR2-knockout control cell lines. In an orthotopic MM xenograft model (U266/NSG), treatment with ROR2-CART significantly reduced tumor burden, improved median overall survival (119 vs. 149 days, p=0.0039), and induced a long-tern curative effect in a subset of mice (25%; 3/12 mice). Importantly, treatment with ROR2-CART was well tolerated in mice, despite comparable gene expression levels and cross-reactivity of the cell product with murine Ror2.

To study the relevance of targeting ROR2 in solid cancers, screened the TCGA database and performed correlation analyses between *ROR2* gene expression and clinical parameters for all 32 projects. Our analyses revealed a significant correlation between clinical parameters and *ROR2* expression in six cancer entities including brain, kidney and stomach cancers. We selected ccRCC for further assessment, due to a strong correlation between *ROR2* expression and disease-specific survival. Using qPCR, we confirmed *ROR2* overexpression in ccRCC patient samples and cell lines, and quantified the expression of ROR2 on the cell surface using dSTORM microscopy.

Co-cultivation with ROR2-CART induced antigen-specific antitumor reactivity against ROR2-positive ccRCC cell lines, but not ROR2-negative reference cells. Similarly, treatment with ROR2-CART significantly increased mOS in a xenograft model of ccRCC (52 vs. 84 days, p=0.0035). As expected, conventional ROR2-CART only elicited a temporary anti-tumor response and did not achieve complete tumor clearance, providing us with a platform to initiate iterative cell product optimization for solid cancers. In a first case study, we explored transcription factor modulation, as well as the combinatorial use of small molecule inhibitors. Our data show that both overexpression of cJUN and the use of the TKI cabozantinib can be used to augment the potency of ROR2-CART, both *in vitro* and *in vivo*.

Collectively, these data show that ROR2 is a relevant target in various hematological and solid tumors. ROR2-positive hematological and solid cancers can be addressed effectively using ROR2-CART without adverse effects in preclinical models.

P0730

Prussian blue nanoparticle-based photothermal therapy generates potent tumor-specific T cells as an adoptive cell therapy

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Tumor-specific T cells that can target multiple antigens have the potential to improve treatment response in solid tumors, complementing chimeric antigen receptor and tumor-associated antigen-specific T cell approaches, which typically target a defined set of antigens. Our group has designed a multi-antigen-targeted T cell therapy to treat cancer, which uses Prussian blue nanoparticle-based photothermal therapy (PTT) to generate tumor-specific T cells. PTT is an experimental tumor treatment approach that involves nanoparticles that convert light-to-heat in the presence of tumor cells, inducing immunogenic tumor cell death. We utilize PTT to promote the release of antigens and adjuvants from tumor cells to prime dendritic cells (DCs), which are then used to stimulate T cells. In recently published studies, we demonstrated that T cells generated using PTT are more specific and cytotoxic toward tumor cells than alternative multi-antigen-targeted approaches, such as freeze-thaw lysis.

Given these observations, we sought to characterize the mechanistic changes contributing to PTT-primed T cell efficacy. We hypothesized that the immunogenicity of PTT contributes to more effective T cell priming. Namely, we hypothesized that PTT provided an advantage in each of the three signals required for T cell activation—antigen presentation to T cell receptors (TCR), costimulation, and cytokine stimulation—promoting tumor-specific T cell expansion. PTT produced a differential cytokine release profile in tumor cells compared to lysate, including differences in IL-1β, IFN-β, and IL-6 secretion, as well as increased immunogenic tumor cell death. We also observed differential cytokine release in DCs primed with PTT tumors compared to lysate, including higher IL-8, IL-1β, and MCP-1 secretion. However, DCs primed with either PTT or lysate produced equivalent costimulation. MHC-presented peptidomic analysis revealed 54 peptides unique to PTT-primed DCs compared to lysate-primed or unprimed DCs. 85% of these peptides overlapped with the tumor peptidome, suggesting that PTT promotes the presentation of distinct and personalized tumor-specific antigens to T cells. This implies that the advantage of PTT stems from differential cytokine stimulation and/or differential antigen presentation by DCs. TCR sequencing analysis demonstrated an increase in T cell Simpson clonality (from 0.04 to 0.14) and maximum productive frequency (from 2.3% to 9.1%) over the manufacturing process, suggesting that our T cell product selectively expands a subset of TCR clones while remaining multi-targeted.

As a proof-of-concept, we expanded T cells generated from PTT-treated patient tumor samples and autologous patient PBMCs. Upon tumor re-challenge, these patient-derived PTT-T cells demonstrated dose-dependent tumor-specific IFN-ɣ secretion (2.5 fold increase) and tumor-specific cytolytic activity (14.8-20.6% killing after 4 h co-culture). TCR sequencing analysis of these T cells also identified an increase in T cell Simpson clonality (0.03 to 0.17) and maximum productive frequency (1.7% to 12.0%).

These findings provide a rationale to further study the mechanisms contributing to differences in DC antigen presentation and T cell expansion, including identifying mechanisms unique to PTT in comparison with alternative multi-antigen-targeted T cell generation approaches. Our results suggest that PTT expands a multi-targeted yet clonal, tumor-specific T cell product, and support the pre-clinical development of our product as a prospective adoptive cell therapy.

P0731

Trogocytosis in CAR-T Cells Targeting Head and Neck Squamous Carcinoma Cells

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Trogocytosis consists of the intercellular transfer of membrane-associated molecules among different cell types. This has been extensively studied in cells of the immune system, and it has been reported that this process can alter the response of the immune cells. Lymphocytes expressing a synthetic chimeric receptor (CAR-T cells) against tumor antigens have shown clinically relevant results against several B-cell leukemia. Nevertheless, the efficacy of CAR-T cells in solid tumors is much lower, and various factors have been proposed to explain limitations in the efficacy of CAR-T cells against cancer cells. This includes the possibility that though a trogocytosis effect, the antitumoral CAR-T cells would acquire antigens from the cancer cell.

Our aim was to determine the existence of a trogocytosis process in CAR-T cells expressing a pan-ErbB ligand during the interaction with head and neck squamous cell carcinoma (HNSCC) cell lines expressing EGFP/Luc (VU-1131 and VU-1365). These cell lines, as primary HNSCC tumors in patients, constitutively express high levels of ErbB molecules, and were used as target cells. After the co-culture of CAR-T cells with the HNSCC cell lines, the percentage of lymphocytes acquiring ErbB1 molecules, defined as $Trog^+$ cells ($CD3^+ErbB1^+EGFP^-$) and $Trog^-$ cells ($CD3^+ErbB1^-EGFP^-$), were tracked at different time points in CAR^+ and CAR^- subpopulations. Additionally, the fratricide killing between $Trog^+$ CAR-T cells was analyzed after 24h of CAR-T/tumor cell co-cultures. In order to prevent trogocytosis, the effect of Lantruculin A (LatA), a drug that avoids the actin polymerization, was analyzed.

Our results demonstrate that trogocytosis was evident in co-cultured CAR-T cells/tumor (40-60% of CAR-T cells showed high levels of ErbB1 molecules on their surface), concomitant with a reduction in the expression of this antigen on the surface of tumor cells. In our experimental model, the trogocytosis process required the direct interaction between the immune and the tumor cells, and this effect had an impact on the survival of CAR-T cells. There was a high trogocytosis-mediated fratricide killing effect in CAR^+Trog^+ cells (60% cell death), compared with CAR^+Trog^- cells. Significantly, when CAR-T cells were treated with LatA for 30 minutes prior to the contact with the tumor cells, a significant reduction in the proportion of $Trog^+$ cells was observed, concomitant with a decrease in the fratricide killing of CAR-T cells. Moreover, both the viability and cytotoxic effect of CAR-T cells against the tumor cell lines was maintained after LatA treatment, particularly when low effector:target cell ratios were used.

In this work, we have described an in vitro model for the study of trogocytosis, in which a significant acquisition of ErbB1 molecules in CAR-T cells, coupled with a down-regulated membrane expression of this antigen in the tumor cells was observed. This process could impair the effectiveness of CAR-T cell immunotherapy in vivo, contributing to the immune escape of the tumor cells. Additionally, our results suggest that this negative effect might be prevented by pre-incubation of the CAR-T cells with LatA.

P0732

Transactivator-free Doxycycline-inducible TRUCKs releasing IL-18 for enhancing antitumoral potential

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Chimeric Antigen Receptor (CAR) T cell therapy has revolutionized type B cancer treatment, while its efficacy remains limited in several lymphomas and solid tumors. Reinforcing conventional CAR-T cells to release cytokines can improve their efficacy but also raises safety concerns. Several strategies have been developed to regulate their secretion using minimal promoters controlled by chimeric proteins harboring transactivators. However, these chimeric proteins can disrupt the normal physiology of T cells, making them less ideal for the generation of Advanced Therapy Medicinal Products (ATMPs). Here, we present the first transactivator-free anti-CD19 CAR-T cells able to control IL-18 expression (iTRUCK19.18) under ultra-low doses of doxycycline (Dox) without altering cellular fitness. Interestingly, IL-18 secretion requires T cell activation in addition to Dox, allowing the external regulation of CAR-T cell potency. This effect was translated into increased CAR-T cell antitumor activity against aggressive CD19+ hematologic and solid tumor models, such as pancreatic cancer (PDAC) and Ewing Sarcoma (ES). In a clinically relevant context, we have generated patient-derived iTRUCK19.18 cells capable of eradicating primary B cell tumors in a Dox-dependent manner. Furthermore, IL-18-releasing CAR-T cells polarized M2 pro-tumoral macrophages towards an M1-antitumoral phenotype in the presence of tumoral PDAC cells, suggesting the potential for modulating the tumor microenvironment. In this line, we have further explored this behavior in 3D multicellular models where iTRUCKS19.18 (+Dox) increased tumor killing in ES-3D spheroids containing M2 macrophages. Additionally, spheroids with M1 and M2 macrophages showed a decrease in the mean expression of CD14, FRb, CD163, and CD209 markers, which are overexpressed in M2-like TAMs when treated with iTRUCKS19.18 (+Dox). In summary, we demonstrated that our platform can generate exogenously controlled CAR-T cells with enhanced antitumor potency in the absence of transactivators.

P0733

Programmable LOGIC AND/OR GATE circuits in living cells

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T cell exhaustion is a progressive dysfunctional state of T cells that occurs in chronic infections and cancer. This condition hinders optimal control of disease, with poor T cell effector function, sustained co-expression of inhibitory receptors (IRs), and altered transcriptional, epigenetic, and metabolic profiles. Exhausted T cells overexpress several transcription factors (TFs) such as MAF, TOX, NR4A2, and EOMES.

Synthetic biology offers the potential to engineer powerful new cellular behaviors, or to reprogram their function with the design and integration of genetic circuits conceived to act in response to endogenous molecular changes.

Genetic circuits that respond to transcriptional dynamics are based on novel synthetic promoters (SPs) targeted by desired, individual TFs whose expression changes accordingly to the cellular state. Synthetic promoters are a convenient alternative to the endogenous counterpart due to the

short size (about 100-150 bp) and lack of crosstalk which confine timing and localization of the downstream genes.

Biological Logic Gating in engineered cells confines the output expression as function of the presence (or absence) of selected inputs. In an immunotherapeutic setting this translates in the tight control of immune response.

In this study, we designed input/output circuits based on Boolean Logic principles that respond to c-MAF and NR4A TFs whose expression progressively increases during T cell exhaustion. By combining novel SPs engineered to respond to MAF and NR4A we developed an AND and an OR gate which demonstrate output activation either when both TFs are upregulated (AND) or when at least one TF increases (OR).

In the AND gate configuration, we used a split synthetic transcription factor (GAL4-VP16) whereby each of the parts (VP16 and GAL4) are fused to N7 and N8 dimerizing tags respectively. VP16-N7 was placed downstream the NR4A-SP, whereas the GAL4-N8 was placed downstream the cMAF-SP. Thus, both NR4A and MAF must be upregulated to trigger the output, the full GAL4-VP16 complex, which in turns activates a downstream promoter.

The OR gate also relies on the split synthetic transcription factor (GAL4-VP16) system. In this setup, VP16-N7 was placed downstream of both the NR4A- and cMAF-SP, while GAL4-N8 was placed downstream of a constitutive promoter, shEF1α. Thus, the full GAL4-VP16 is formed when one among NR4A or MAF is upregulated.

Transfections on HEK 293FT cells demonstrated that the fluorescence intensity under the logical TRUE condition was significantly higher than under the logical FALSE condition, validating the functionality of these logic gates. Due to the programmability of Synthetic Promoter switches, constructs based on logic gate rules could serve as programmable therapeutic devices. This approach could establish a versatile and powerful platform for regulating T cell exhaustion and potentially reversing its progression.

In conclusion, our programmable AND/OR gate circuits represent a significant advancement in synthetic biology, with potential applications in therapeutic strategies for chronic infections and cancer. By precisely controlling cellular behavior through Synthetic Promoters responsive-sensible to specific TFs, we can develop innovative treatments to address T cell exhaustion and improve desired outcomes.

P0734

Reduction of muscle fibrosis by FAP-CAR T cells enhances rAAV gene transfer efficiency in a murine model of Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is a progressive muscle disorder that weakens skeletal and cardiac muscles and causes fibrosis in these tissues. A rapid and intense fibrotic process develops in the DBA2mdx mouse model of severe DMD, visible at 2 months of age, and characterized by dense collagen deposits in skeletal muscles, with over-expression of the FAP and Col3a genes compared to the aged-matched DBA2 littermates. It was hypothesized that fibrosis may limit the

effectiveness of gene transfer therapy in DMD but direct evidence is lacking and strategies to reduce skeletal muscle fibrosis are limited. Because FAP-specific CAR-T cells were shown to reduce a rapidly-ocurring drug-induced cardiac fibrosis model in normal mice, we tested whether FAP-specific CAR-T cells could also reduce the broad and spontaneously occurring skeletal muscle fibrosis in DBA2mdx mice. A FAP-CAR lentiviral vector (LV) encoding a third-generation CAR with an mFAP-specific ScFv was generated and validated on FAP-overexpressing 3T3 cells. The FAP-CAR LV was used to generate FAP-specific cytotoxic T cells from DBA2 spleen cells. Two consecutive administrations of FAP-CAR T cells to DBA2mdx mice reduced the histological and molecular biomarkers of fibrosis, as measured 2 weeks post-cell injection. Mice treated by FAP-CAR-T cells and receiving an intravenous administration of rAAV9 encoding a microdystrophin (5xE12 vg/Kg), demonstrated an increased gene transfer efficacy as demonstrated by higher viral genome and transgene expression in TA and EDL muscles compared to controls. These results demonstrate for the first time that fibrosis acts as a restriction to rAAV gene transfer and provide a novel therapeutic option to treat muscular dystrophies by combining gene therapy and immunotherapy.

P0735

Generation of inducible signal3-based CAR-T cells for cancer treatment

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Clinical efficacy of CAR-T cells in patients with solid tumors has been lower than expected. A key limitation in the setting of solid tumors is a lack of expansion and survival of CAR-T cells. Providing the signal 3 to CAR-T cells has been proposed as a strategy to overcome this limitation. Most of the CAR constructs studied in the clinic include only the first and second signal, while strategies to provide signal 3 are typically focused on constitutive interleukin signalling, which can generate toxicities. Here, we hypothesized that inducing signal 3 in CAR-T cells upon antigen recognition through cytokine receptor activation motifs from the IL-7Rα will increase T-cell activation and overall persistence.

First, we designed chimeric cytokine receptors (CCR) by coupling a scFv to the intracellular domain of the IL-7Rα including either the IL-7Rα or CD8α transmembrane domain. IL7-CCRs were efficiently expressed on T-cell surface, and further characterized in single transduction or co-transduction with a first-generation CAR. In single transduction, CCRs were unable to induce STAT5 signalling after antigen recognition, as compared to a constitutive IL-7R able to phosphorylate STAT5 autonomously. In co-transduction with a CAR, the CCRs did not provide a cytotoxic nor a proliferative advantage.

We hypothesized that the steric conformation of the scFv, bigger than the wild-type IL7-Rα extracellular domain, may impede dimerization and subsequent signaling. To validate this, we replaced the scFv by the extracellular domain of the PD1 receptor, reducing by half the size of this region. After PD-L1 recognition, the PD1-IL7 CCR failed to phosphorylate STAT5 or to enhance proliferation of these cells, suggesting that this might not represent the main problem underlying the absence of signaling.

Next, we explored the addition of specific IL-7Rα signaling motifs within an ICOS or CD28-based second-generation CAR constructs. Within the intracellular domain of the IL-7Rα we find the Box1, interbox and Box 2 motifs that mediate JAK activation. Then, STAT5 is activated by some tyrosine residues located at the end of the cytoplasmatic tail. We generated a panel of constructs including these signaling regions in different spatial conformations. Once expressed in T-cells, we observed differences in terms of expression and cytotoxicity *in vitro* among the different constructs. We could not detect a clear upregulation in phosphorylation of STAT5 after antigen recognition, probably due to background STAT5 signalling from the CD3ζ domain. Using an *in vitro* model of continuous antigen exposure, some IL7R-based constructs revealed enhanced cytotoxicity compared to the control. *In vivo*, IL7-based CARs behaved similarly to the control CAR, showing no clear advantage in terms of antitumor activity.

As previously published, diverse IL-7R conformations may signal constitutively once expressed in T-cells. However, we have demonstrated that, if these signalling domains are expressed in an inducible conformation, they lack STAT5 signalling. Further characterization of the CCRs in terms of signaling upon homodimerization, as well as heterodimerization either with the endogenous or overexpressed IL-2Rɣ will be presented. We believe that these results could shed light on dynamics of cytokine signalling and allow the design of an inducible CCR.

P0736

CD8-targeted anti-CD4CAR lentivectors generated *in vivo* eliminate malignant CD4+ T cells in a murine T cell lymphoma preclinical model

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Angioimmunoblastic T cell lymphoma (AITL), one of the most prominent peripheral T cell lymphomas, represents a rare complex malignancy affecting mostly elderly, with no specific treatments available and a poor survival outcome. The optimal management of AITL represents an unmet medical need as its low prevalence makes it difficult to design and evaluate novel therapeutic strategies.

However, to address this medical challenge, we have generated a unique preclinical mouse model for AITL by overexpressing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) - a glycolytic enzymes emerging now as a key player in T cell survival, development and function - exclusively in T cells. This resulted in a T cell lymphoma closely mimicking the clinical and pathological features of human AITL disease.

Since in human AITL, the drivers of the malignancy are the $CD4^+$ follicular helper T cells, we wanted to generate CD8+ T cells expressing a chimeric antigen receptor (CAR) that would eliminate the CD4+ malignant T cells. For this purpose, we designed a lentiviral vector (LV) coding for an anti-mCD4 CAR and pseudotyped with modified paramyxovirus envelope glycoproteins, in order to allow its exclusive entry into CD8+ T cells (anti-CD4CAR CD8-LVs), thereby avoiding CAR expression by their malignant counterpart CD4 T cells. *In vitro* analysis of the murine AITL

biopsies transduced with the anti-CD4CAR CD8-LVs showed expansion of these tumor infiltration CD8 T cells, while the CD4 neoplastic Tfh-like cells were almost completely eliminated as compared to control transductions with GPF encoding CD8-LVs. These *in vitro* results encouraged us to evaluate the anti-CD4CAR CD8-LVs *in vivo* by direct injection into the bloodstream of our preclinical mAITL model. Also in this case, high CAR-expression levels in CD8+ T cells were achieved and malignant CD4+ T cells were eliminated from the mAITL lymphoma, following anti-CD4CAR CD8+ T cells expansion upon encounter with the CD4 receptor converting them into potent cytotoxic T cells. Consequently, injection of CD8-LVs coding for anti-CD4CAR resulted in highly increased survival (80%) of the mAITL recipient mice as compared to the control group injected with GPF encoding CD8-LVs.

In summary, the *in vivo* generation of functional anti-CD4CAR CD8 effector T cells, decreased significantly the number of CD4+ neoplastic T cells from the tumors, which correlated with increased survival of the mAITL preclinical model. This might offer a new therapeutic perspective for patients suffering from a CD4-driven T cell lymphoma. In addition, *in vivo* CAR T cell generation would surmount the main problems of current CAR T cell therapy, such as the time-consuming, labor-intensive and costly process of *ex vivo* CAR T cell therapy.

P0737

Tolerogenic IL-10-engineered dendritic cell-based therapy to restore antigen-specific tolerance in autoimmune diseases

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Antigen- (Ag-)specific therapies represent a promising avenue for addressing the unmet medical need of patients with autoimmune diseases. Tolerogenic dendritic cells (tolDC) play a crucial role in promoting tolerance and represent the cells of choice to fulfill the goal of restoring Ag-specific tolerance in autoimmunity. Effective DC-based therapy should dampen autoreactive T cell responses and restore long-term tolerance *via* Ag-specific regulatory T cell induction**.** We generated tolDC by genetic engineering of human monocytes with lentiviral vectors (LV) co-encoding for Ag-derived peptides and IL-10. Our LV platform enforces presentation of the encoded peptide in the context of HLA-class II molecules, thus allowing efficient Ag presentation by engineered DC to $CD4^+$ T cells. We previously demonstrated that the resulting engineered DC (designated $DC^{IL\text{-}10/Ag}$) constitutively secrete IL-10, acquire a tolDC-like phenotype, efficiently downregulate Ag-specific T cell responses and promote *in vitro* Ag-specific $CD49b^+LAG\text{-}3^+$ Type 1 T regulatory (Tr1) cells in healthy subjects carrying the appropriate serotype. To validate our platform for tolerance induction in the context of autoimmunity, we chose as model diseases celiac disease (CD) and type-1 diabetes (T1D). In CD the deamidated 17-mer of α-gliadin epitope is recognized as peptide inducing pathogenic T cell response in HLADQ2.5 patients. Conversely, autoreactive T cells with multiple Ag specificities have been involved in the attack to pancreatic b cells in T1D. To select the most cross-reactive auto-Ags in T1D, we screened peripheral T cell response to previously described HLA-DQ8-restricted epitopes by IFNg ELISPOT and selected Hybrid-insulin-peptide 11 (HIP11), Neuropeptide $Y_{60\text{-}75}$ (NPY), and a mimotope of $InsB_{9\text{-}23}$ (InsMIM) as the most HLA-DQ8 cross-reactive peptides for tolDC engineering. $CD14^+$ cells

from HLA-DQ2+ CD subjects and HLA-DQ8+ T1D patients were transduced during DC differentiation with LV encoding for the selected epitopes with IL-10. The resulting $DC^{IL\text{-}10/Ag}$ were efficiently transduced, acquired the expected tolDC phenotype, secreted IL-10, and inhibited Ag-specific IFNg release by autologous $CD4^+$ T cells. Moreover, the frequency of Tr1 cells in culture with $DC^{IL\text{-}10/Ag}$ was increased as compared to control DC in patients with detectable Ag-specific reactive T cells, indicating that engineered tolDC from patients with autoimmunity can potentially promote tolerance to selected autoAgs. However, the efficiency of functional tolDC induction in patients was lower than that achieved with $CD14^+$cells from healthy subjects, suggesting that the mechanisms controlling the acquisition of tolerogenic functions during tolDC differentiation might be defective in monocytes from patients with autoimmunity. Preliminary data from *in vitro* studies of monocytes of subjects with autoimmune diseases suggest that tolDC from autoimmune patients might be less potent inducers of Tr1 cells than those differentiated from healthy subjects. Studies to identify the key molecular and metabolic switches controlling IL-10-producing DC differentiation are currently ongoing. Collectively our results indicate that $DC^{IL\text{-}10/Ag}$ represent a platform to induce stable Ag-specific tolerance with the potential to control T cell response to pathogenic Ags in autoimmune diseases.

P0738

Enhancing CAR-T Cell Therapy Through PD-1 deletion and Controlled IL-15 Expression

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Adoptive Cell Therapy (ACT) with genetically modified T cells expressing **Chimeric Antigen Receptors** (**CAR**) is promising for refractory leukemia or lymphoma. However, CAR-T therapy faces challenges like toxicity, tumor microenvironment inactivation, and low cell persistence. This work aims to develop a gene editing strategy to generate CAR-T cells that overcome inhibitory signals from **Programmed death-ligand 1** (**PD-L1**) expression in tumor cell. The initial approach involved **PD-1 deletion in CAR-T cells** and a detailed characterization of their phenotypic and metabolic features. The CAR utilized in this work, CAR **ARI-0001**, is a second-generation CAR specific for CD19. PD-1 elimination was achieved through CRISPR/Cas9 ribonucleoprotein (RNP) delivery targeting exon 1 of the PDCD1 gene. Delivery of gRNA and Cas9 through RNP electroporation enables highly efficient PD-1 depletion (80%) without significant alterations in T cell phenotype or generating large deletions. Subsequent cellular and metabolic characterizations of CAR-T PD-1 KO cells revealed an increase in exhaustion markers, reduced proliferative capacity and lower spare respiratory capacity (SRC), indicating decreased mitochondrial oxidative potential. Despite this reduction in metabolic fitness, compared to ARI CAR-T cells, ARI PD-1 KO CAR-T cells presented an increase in memory phenotype and enhanced persistence and cytotoxicity when confronted to PD-L1-expressing tumor cells. These findings indicate that,

although PD-1 KO improves CAR-T cell persistence in the presence of PD-L1, it simultaneously induces a reduction in cell fitness that should be improved to enhance the therapeutic potential of these strategy. To achieve this goal, we proposed a **controlled expression of IL-15**, a cytokine that improves T cell proliferation and metabolism. A CRISPR/Cas9-Homology-Directed Repair platform (**HDR**) was designed to **express IL-15 from the PD-1 endogenous promoter**. Various DNA delivery methods were evaluated, with AAV6 proving to be the most efficient for primary T cells, achieving around 60% integration efficiency. The platform combined RNPs for CRISPR/Cas9 delivery and AAV6 for IL-15 cDNA delivery, to generate **pdTRUCKIL-15 cells.** As expected, IL-15 expression in pdTRUCKIL-15 cells followed the PD-1 expression profile. pdTRUCKIL-15 cells showed enhanced proliferation, reduced pro-apoptotic protein BIM, increased anti-apoptotic protein Bcl-xL, improved metabolic characteristics, and higher lytic capacity compared to CAR-T PD-1 KO and CAR-T WT cells. In summary, in this work we have demonstrated that PD-1 deletion in CAR-T cells enhances their activity against PD-L1+ tumor cells but reduces metabolic potential and proliferative activity. We also showed that controlled IL-15 expression under PD-1 endogenous promoter reverses these deficiencies, making pdTRUCKIL-15 cells better suited to confront PDL-1+ tumors, ensuring prolonged survival and persistence. This strategy represents a significant advance in CAR-T cell engineering, offering a platform that could be applied to other PDL-1+ cancer types.

P0739

Base edited CAR T cells for combinatorial strategies against acute myeloid leukaemia

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Acute myeloid leukaemia (AML) is an aggressive, life-threatening disease with limited curative options. Chimeric antigen receptor (CAR) T cell approaches are under investigation but disease heterogeneity is problematic and targeting of multiple antigens will be required to secure complete molecular remission. Suitable candidate antigens for combinatorial approaches include the transmembrane receptor Siglec-3 (CD33), C-type lectin-like receptor-1 (CLL-1) and to a lesser extent CD7, an Ig-superfamily molecule that is highly expressed on T and NK cells and certain myeloid leukaemias. Anti-CD7 CAR T cells are already being investigated to treat T cell malignancies and have been reported to mediate profound lymphodepletion and bone marrow aplasia, including in an ongoing trial of base edited BE-CAR7 T cells in paediatric T-cell acute lymphoblastic leukaemia (T-ALL) at our centre. Here we report the development and modelling of 'universal' donor CAR T cell banks targeting CD33, CLL-1 and CD7, for use alone or in combination, after suitable base editing to allow co-infusion whilst securing protective effects against fratricide and agents such Alemtuzumab, an anti-CD52 lymphodepleting monoclonal antibody used prior to CAR T cell infusion. Base edited "universal" $TCR_{a/b}^{-}$ $CD7^{-}$ $CD52^{-}$ BE-CARCLL-1, BE-CAR33 and BE-CAR7 effectors were generated from peripheral blood mononuclear cells by lentiviral transduction following activation, and electroporation with base-editor mRNA alongside multiplexed base editing single guide RNAs. Disruption of *TRBC1/2* aimed to prevent unwanted $TCR_{a/b}$ mediated allo-effects against recipient cells (graft-versus-host disease, GvHD), or populations of human leukocyte antigen (HLA)-mismatched co-infused allogeneic CAR T cells. Disruption of *CD7* was included to enable BE-CAR7 production and allow co-infusion of other BE-CAR products, and *CD52* editing conferred resistance to Alemtuzumab. Cells were characterised by flow cytometry and molecular mapping, and *in vitro* cytotoxic

functions were tested using ^{51}Cr release assays against HL-60, Molm-14, U-937 and Kasumi-3 cell lines. BE-CARCLL-1 and BE-CAR33 targeting effects were also demonstrated against CLL-1^{+}CD33^{+} primary AML sample *in vitro* after overnight co-culture. In humanised mice there was appropriate reduction in bioluminescent signals for each CAR T cell product independently, confirming *in vivo* anti-leukemic effects over a three or four week period compared with untransduced controls ($p<0.005$). Compatibility of BE-CAR7 and BE-CAR33 after removal of CD7 was also confirmed in a humanised PDX model. CRISPR-engineered CLL-1^{-}CD33^{+} and CD33^{-}CLL-1^{+} HL-60 cell lines were generated to model disease heterogeneity. When engrafted into NSG mice, leukaemic burden was reduced in bone marrow sites after combined BE-CARCLL-1 and BE-CAR33 dosing compared to mice receiving either product in isolation ($p<0.01$). No difference was noted in survival after tumour re-challenge mimicking heterogenous disease recurrence. Therapeutic strategies envisage 'pick & mix' applications of universal CAR T cell combinations determined by patient-specific target selection after flow-based disease immunophenotyping. Combinations of fratricide-resistant, lymphodepletion insensitive, co-infusion compatible CAR T cells would be harnessed for leukaemic eradication and deep preparative conditioning ahead of programmed, donor-derived reconstitution following allogeneic haematopoietic stem cell transplant (HSCT).

P0740

N-glycosylation inhibition hinders immunosuppressive tumor microenvironment cells improving CAR T cell efficacy

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Adoptive transfer of CAR T cells demonstrated impressive results against B-cell malignancies, but still limited efficacy against solid tumors. In this context, multiple challenges need to be overcome, including poor tumor recognition and strong immunosuppression within the tumor microenvironment (TME). Our Unit has recently reported that pharmacological inhibition of N-glycan synthesis in cancer cells increases CAR T cell efficacy by improving tumor recognition and preventing T cell exhaustion. In this project, we investigated the role of N-glycosylation blockade on TME cells in the context of colorectal cancer (CRC) and pancreatic adenocarcinoma (PDAC)-derived liver metastases, exploiting CEA-specific CAR T cell therapy.

To understand the effect of N-glycosylation blockade on TME cells (both M2-like macrophages, M2-M and Hepatic stellate cells, HepSCs), we analysed the phenotypic and transcriptional profile and we performed *in vitro* functional assays, such as tripartite co-cultures, suppressive assays and released-cytokines analysis. *In vitro* studies revealed that N-glycosylation inhibition abolishes the ability of both TME cells to restrain T cell proliferation and increases the elimination of cancer cell lines and patient-derived tumor organoids (PDOs from CRC-liver metastases and from primary PDAC). Interestingly, these effects were associated with profound phenotypic and transcriptional changes in M2-M and HepSCs. In particular, the treatment was able to inhibit M2-polarization in terms of surface markers expression, IL-10 secretion and gene expression profile, and was shown to hinder the activation of HepSCs.

Moreover, to evaluate the effect of N-glycosylation inhibition on TME cells *in vivo*, we exploited HSPC-SGM3 humanized mouse model, wherein infusion of human CD34+ cells facilitates reconstitution of a human immune system. Then, mice are engrafted intra-liver with tumor cells and treated with CEA CAR T cells. Interestingly, in this *in vivo* model, the presence of human immune cells helps recreate an immune TME more representative of the human disease. Importantly, using these mice we observed that N-glycosylation inhibition improves CEA CAR T cells fitness, increasing their antitumor activity in terms of survival. This effect is associated with a modulation of human immune population recapitulated in the liver TME and in particular with the reduction of pro-tumorigenic IL1B+ TAMs and the loss of inhibitory interactions between ligand/receptor pairs (from scRNAseq analysis).

Overall, these data suggest that blocking N-glycosylation can help overcome multiple barriers that currently limit CAR T cell efficacy in solid tumors, acting not only on tumor cells, but also on immunosuppressive tumor microenvironment cells.

P0741

Novel SdAb-based CAR-T cells against BCMA with improved therapeutic efficacy for Multiple Myeloma

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Multiple myeloma (MM) is a clonal plasma cell malignancy accounting for approximately 10% of all hematological cancers. Despite advancements in personalized, antibody-based, and cell-based immunotherapies, patients with relapsed or refractory disease still face poor prognoses, highlighting the urgent need for novel therapeutic strategies. This research explores an innovative approach to cancer treatment through Chimeric Antigen Receptor T-cell (CAR-T) therapy, focusing on the potential benefits of utilizing single-domain antibodies (sdAbs) to enhance therapeutic efficacy.

After phage display, sdAbs specific for BCMA and corresponding to different families were identified. SdAbs (one per family) were selected for further characterization, and three CAR constructs were selected based on their affinity, binding kinetics, absence of tonic signaling, and specific activation in response to BCMA+ cells. These selected constructs were used to generate CAR-T cells, which were evaluated in vitro and in vivo, using an scFv-based CAR-T as a control. All CAR-T cells presented high cytotoxic activity and produced high levels of cytokines (IFNγ, IL-2, and TNFα) in response to MM cell lines expressing BCMA.

The best-performing sdAbs were incorporated into CAR constructs by replacing the single-chain variable fragment (scFv) sequence in a second-generation 4-1BB construct. These sdAb-based CAR constructs were then characterized in vitro using the Jurkat TPR (Triple Reporter) system, which allows for the assessment of binding properties and downstream signaling efficacy.

Following in vitro characterization, sdAb-based CAR-T cells were produced via lentiviral transduction of activated T cells from healthy donors. These engineered CAR-T cells were then evaluated for their cytotoxicity and phenotypic profiles using the Bright-GloTM Luciferase Assay System.

To assess in vivo antitumoral efficacy, the sdAb-based CAR-T cells were tested in immunodeficient NSG mice xenografted with MM1S cells, a human multiple myeloma cell line. Comparative analysis with second-generation scFv-based CAR-T cells (ide-cel) revealed that sdAb-based CAR-T cells exhibited superior cytotoxicity and cytokine production (including IFNγ, IL-2, and TNFα) in response to MM1S cells. Furthermore, the sdAb-based CAR-T cells demonstrated enhanced antitumoral efficacy and improved survival rates in vivo, as evidenced by luciferase imaging and survival analysis in NSG mice.

In conclusion, this research successfully identified and characterized multiple sdAb-based CAR-T cells with different affinities, demonstrating their potential to surpass the antitumoral efficacy of traditional scFv-based CAR-T cells. These findings underscore the promise of sdAb-based CAR-T cell therapy as a novel and effective treatment for BCMA-positive MM and potentially other hematological malignancies. The enhanced cytotoxicity and antitumoral efficacy observed in preclinical models pave the way for further clinical development and potential therapeutic application, offering new hope for patients with relapsed or refractory multiple myeloma.

P0742

Generation of T cell receptor transgenic regulatory T cells for adoptive therapy in Type 1 Diabetes

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CD4+CD25^{bright}CD127^{low}FOXP3+ regulatory T cells (Tregs) hold promise as “living drug” to restore immune tolerance in autoimmune disorders, including type 1 diabetes (T1D). However, Early-phase clinical trials using a single infusion of polyclonal Tregs have demonstrated a limited therapeutic effectiveness in patients with T1D. We aim to address these limitations by generating beta cell antigen specific Tregs. We first focused on a published TCRs specific for the IGRP$_{265-273}$/HLA-A*0201 complex. After validating the specificity and biological functionality of IGRP-specific TCR-edited T cells against both HLA-*0201 positive cell lines loaded with the related antigen and HLA-2A mouse islets expressing the same human epitope, the class I-restricted TCR was transduced into magnetically sorted CD4+CD25^{high}CD127^{low} Tregs after disrupting the endogenous TCR through CRISPR/Cas9 technology targeting the TRAC and TRBC1/2 loci simultaneously. This approach avoids TCR alpha-beta mispairing and maximizes TCR expression and function, effectively redirecting Treg specificity permanently. Lentiviral-infected Tregs expanded at similar rates to mock-infected Tregs and maintained the phenotypic characteristics of Tregs in vitro, as demonstrated by enrichment of the specific Vbeta. However, our IGRP-specific Tregs were not able to efficiently bind IGRP-specific dextramers or undergo antigen-specific activation. In contrast, edited IGRP-specific conventional T cells (Tconv) showed the ability to bind the relative dextramer only in their CD8+ subset, not in the CD4+ subset. This suggests that the activation of the IGRP-specific TCR in our engineered Tregs might depend on the expression of the CD8 co-receptor. In parallel we started hunting for novel TCR specific for the GAD65$_{114-123}$/HLA-A*0201 complex, a well characterized, highly prevalent and immunogenic peptide/MHC complex in patients with T1D. To isolate, sequence, and clone GAD65-specific TCRs, we performed clonal tracking of the TCR repertoire of HLA-A*0201 conventional T cells

stimulated with autologous APCs loaded with $GAD65_{114-123}$ peptide. Post-stimulation, the TCR repertoire narrowed over time, as demonstrated by the enrichment of specific Vbeta. Moreover, T cells exhibited in vitro responses to $GAD65_{114-123}$, including cytokine production (IFN-γ), dextramer positivity, proliferation, and up-regulation of CD137. We performed high throughput TCR sequencing of T cells collected after each stimulation to identify the alpha and beta chains of $GAD65_{114-123}$-specific TCRs. We currently identified four candidate TCRs for further testing. In conclusion, we generated IGRP specific Tregs demonstrating our capacity to efficiently re-direct antigen specificity of polyclonal Treg. This strategy will be applyed to the generation of GAD65 specific Tregs to generate a novel Treg product to be developed in the perspective of improving adoptive Treg therapy for patients with T1D.

P0743

Cell-based potency assays for CAR T-cell drug products: T2EVOLVE focuses on the need for standardization

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According to the International Conference on Harmonization (ICH), potency is defined as "the quantitative measure of biological activity based on the attribute of the product that is linked to the relevant biological properties". An ideal potency assay should be able to detect quantitative changes in measured parameters with accuracy, sensitivity and specificity based on the product's mechanism of action (MoA). Moreover, in a production field for therapeutic use, potency assays should be designed to assess the quality, efficacy and stability of the drug product (DP), supporting its release for clinical administration.

In the field of cancer immunotherapy, T cells genetically modified to express a chimeric antigen receptor (CAR T-cells), exert their activity through simultaneous functions, requiring users to develop *ad hoc* potency assays to ensure their functionality and biological activity.

T2EVOLVE includes academic and industry members in cancer immunotherapy started in 2021, under the European Union's Innovative Medicines Initiative (IMI) with the aim to accelerate the development of engineered T-cell therapies increasing patients' access, by providing guidance, standards and novel models.

The Consortium has investigated which methods for potency evaluation and instruments are used among academic and industrial members. The majority of participants measure potency through killing assay method (evaluating killing efficacy of CAR T-cells) by flow-cytometry approach (60%). However, also luminescence (10%) and real-time cytotoxic assays are used (20%). Based on these findings, the different cell culture-based biological assays were described, and the different read-out methods have been discussed and compared, focusing on their *pro* and *cons* features. In particular, flow cytometry allows to discriminate between target and effector cells at different E:T ratios by using specific antibodies, or by the evaluation of fluorescent protein expressed by the target cells (i.e. GFP). As advantage, this approach gives the possibility to clearly distinguish between live and dead cells by chemical compounds (i.e. 7-AAD). Additionally, T cells can be further characterized by their expression of activation or exhaustion markers. However, this method is labour-intensive and analysis can only be performed at selected time points, and samples recovery is not possible. Real-time cytotoxic assays, which can be based on fluorescence cell imaging or impedance methods, enable continuous monitoring of CAR T-cell killing activity on target cells. These assays provide valuable insights into the efficacy of CAR T-cells. For both the techniques, although allow the differentiation between effector cells and target cells, a detailed phenotypic cell characterization is not possible. As third approach, i.e. the potency evaluation by luminescence assay, is a quite simple read-out which is readily accessible, as the necessary plate-readers are widely available in many laboratories. Although the assay allows for fast, high-throughput screening, it does not allow for further analysis to characterise T cells and it may be difficult to discriminate between effector and target cells. Based on all the above considerations, together with the fact that many different products are under development, there is an evident need to identify an optimal approach to standardize potency assay across different DPs and different indications.

P0744

Clinical and translational findings following MuSK-CAART infusion without preconditioning in patients with Myasthenia Gravis (MuSCAARTes™ trial)

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Muscle-specific tyrosine kinase (MuSK) myasthenia gravis (MG) is a rare but potentially severe disease, in which patients develop pathogenic autoantibodies that specifically target the MuSK protein in the neuromuscular junction. A Phase 1, open-label, study of autologous muscle-specific tyrosine kinase chimeric autoantibody receptor T cells (MuSK-CAART) was conducted to evaluate the safety and early activity of MuSK-CAART in patients with anti-MuSK antibody positive MG (NCT05451212). By binding B cells expressing the MuSK specific B cell receptor (BCR) through the MuSK autoantigen expressed on its targeting domain, MuSK-CAART is designed to specifically eliminate only autoreactive B cells. We report on the first month of translational data associated with four patients dosed with MuSK-CAART without preconditioning at two different dose levels: $5x10^8$ (n=2) and $2.5x10^9$ (n=2) transduced CAART cells. The manufactured cell product ranged from 42% to 70% CAAR positive cells and consisted of a mixed phenotype with three patient products predominantly $CD4^+$ (65% to 89%) and one predominantly $CD8^+$ (85%). All transduced cells were predominantly effector memory phenotype ($CCR7^-CD45RA^-$). The cell product from all four patients effectively lysed anti-MuSK BCR expressing target cells in vitro. MuSK-CAART cells were detected in the circulation of all patients by flow and qPCR with.

Persistence C_{max} ranged from 4 to 29 days post-infusion across the four patients and reached levels in line with those observed in studies of CD19 chimeric antigen receptors in oncology in all four patients. Persistence area under the curve over the first month (AUC_{29d}) was not different between the two dosing cohorts. Baseline level of anti-MuSK autoantibodies demonstrated a high degree of variability amongst patients. Three out of the four patients had low baseline autoantibodies. Two out of the four patients exhibited a >20% drop within the first month after infusion. A mild Grade 1 cytokine release syndrome (CRS) was observed in a patient in the higher dose cohort without any other events of neurotoxicity or serious adverse events among the treated patients. MG activities of daily living (MG-ADL) exhibited clinically meaningful improvements withing the first month for two out of four patients, and remained stable in the other two patients. These data suggest that MuSK-CAART therapy without preconditioning is well tolerated and efficacious in treating MuSK-associated MG. Additional longitudinal clinical and translational MuSK-CAART data will provide more context to interpret these preliminary findings.

P0745

Validating an *in vitro* model for macrophage polarisation to develop a CAR-macrophage therapy for hepatocellular carcinoma.

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Hepatocellular Carcinoma (HCC) treatment relies heavily on surgical resection. Issues with recurrence and generally poor 5-year survival rates mean new treatments are necessary to tackle to disease. Chimeric-Antigen-Receptor (CAR) based T cell therapies have shown good efficacy in haematological cancers but demonstrate limitations against solid immunologically “cold” tumours such as HCC, due in part to poor infiltration into the Tumour Microenvironment (TME). Macrophages represent up to 50% of cells in tumour masses and are usually polarised towards a pro-tumoral M2 phenotype. As such, they present a compelling target to activate an anti-tumour response by repolarising them towards a pro-inflammatory M1 state, using newly developed CAR-Macrophage therapies. An in vitro macrophage model was developed to serve as a platform for CAR engineering. The THP-1 monocytic cell line was differentiated in naïve M0 macrophages with PMA (150nM) and subsequently polarised with either IFN-g (20ng/ml) and LPS (10pg/ml), or IL-4 (20ng/ml) and IL-13 (20/ng/ml), to achieve M1 and M2 cells respectively. RT-qPCR was performed to assess whether these cells showed hallmark gene expression. Cells were also independently co-cultured with HEPG2 to assess gene expression in the presence of liver tumour cells and treated with a clinically proven CAR construct as a proof of concept. Finally, treatment with IFN-g was used to demonstrate a positive therapeutic polarisation towards the M1 phenotype. M1 cells expressed significant upregulation ($P<0.05$) of M1 related cytokines: CXCL10, IL-1B, and TNF-a, while M2 cells showed significant upregulation of fibronectin. Treatment of M2 cells with IFN-g resulted in upregulation of M1 markers IL-8, TNF-a, CXCL10, and downregulation of M2 markers FN1, IL10, EGF, VEGF. CAR transduction of M2 cells achieved 48% efficiency and unexpectedly resulted in downregulation of both M1 and M2 markers. In summary, this in vitro model has been validated as a platform for testing of CAR-M designs, with a view to generating a novel intervention for HCC and further solid tumours.

P0747

Ultrafast cell functionalization via bioorthogonal conjugation: a new approach for cell therapy

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Here, we described an ultrafast technology for functionalizing cell membranes with various ligands. This technology is based on a covalent coupling reaction utilizing diazonium salts to facilitate aromatic electrophilic substitution on tyrosine phenol groups in cell membrane proteins. Currently, metabolic glyco-engineering (MGE) is the predominant method for modifying cell surfaces. It requires the incubation of the cells with a sugar that is modified with a bioorthogonal chemical function (such as an azide or alkyne). Once internalized, the sugar is incorporated into the oligosaccharides on the cell membrane, allowing the attachment of the desired ligand using click chemistry. Although effective, MGE requires co-culturing cells with a non-natural sugar for several days, which can be toxic to some cell lines and also results in low incorporation rates. Therefore, optimizing membrane protein functionalization is crucial for advancing such new cell therapy strategies. Our novel one- or two-step process achieves bioconjugation of fluorophores, carbohydrates, biotin, or other biomolecules in only a few minutes, without observed toxicity or concentration limitations, unlike MGE. We validated this approach using both adherent (HeLa) and suspension (EXPIf, Jurkat, NK92, DC2.4, and HMC3) cell lines. Using DC2.4 cells, we demonstrated that the two-step process involving azido diazonium salts and cyclooctyne-fluorescein resulted in an increased number of bioconjugated ligands compared to the MGE strategy. Additionally, our method reduces the reaction time from 72 hours to less than 1 hour and allows the modulation of the number of grafted molecules, a feature that is not possible with MGE. Importantly, we also observed that cell cultures remained unaffected for 48 hours post-bioconjugation, maintaining their division profiles. To further elucidate the potential of tyrosine bioconjugation for cell membrane modification in cell therapy, functional, targeting and transcriptional analyses are underway, both *in vitro* and *in vivo* models. This innovative bioconjugation technique holds significant promise for enhancing the efficiency and safety of cell therapy applications.

P0748

Improving migration, tumour accumulation, and persistence of tumour-infiltrating lymphocytes (TILs) in ovarian cancer

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The deprived endogenous co-stimulation and the hindered T-cell trafficking into the tumours constitute the principal hurdles for ovarian cancer (OC) immunotherapy. We hypothesise that both axes can be tackled using TIL modification with the synthetic co-stimulatory antigen

receptor (CoStAR) to provide stimulation and chemokine receptors to enhance migration towards OC. Primarily, the expression of chemokine receptors on the surface of TILs and the presence of the respective chemokines in ascites of OC patients were analysed using flow cytometry and meso scale discovery (MSD), respectively. The surface staining of chemokine receptors identified CXCR2, CXCR5, CX3CR1, CCR1 and CCR3 as candidates for TIL transduction since their expression was low (0-20%). In addition, the analysis of chemokine concentration in OC ascites showed high amounts of CX3CL1 (12 804.71 ±58 943.7 pg/mL), intermediate levels of CCL11 (1142.12 ±2680.5 pg/mL) and CXCL13 (722.20 ±2192.5 pg/mL) and low levels of CXCL8 (253.39 ±342.6 pg/mL) and CCL5 (226.91 ±640.08 pg/mL), which are ligands of CX3CR1, CCR3, CXCR5, CXCR2 and CCR1, respectively. Collectively, the low expression of CXCR2, CXCR5, CX3CR1, CCR1, and CCR3 on the surface of TILs, and the detectable secretion of the matched chemokines in the tumour microenvironment, suggested that these receptors are good candidates for TIL engineering. Following healthy donor (HD) T cell and TIL transduction with lentiviral vectors (LVV) of the identified targets, the transwell migration assay was used to assess the migratory capacity of the cells *in vitro*. The migration assessment revealed that CXCR2, CXCR5 and CCR1 transduced cells outperformed when compared to MOCK cells. More specifically, superior migration was observed by CCR1 and CXCR5 transduced T cells towards the corresponding chemokines (30589.73 ±10500.0, 6669.98 ±10478.1, respectively) when compared to MOCK T cells. These enhanced migration attributes are currently being evaluated in TILs. CXCR2 TILs presented significantly greater migration rate (6036.0 ±1595.2) than MOCK TILs (-5754.0 ±2045.0) when IL-8 was present. CoStAR/CXCR2 TILs showed the highest migration towards IL-8 containing ascites (57198.0 ±2672.9) compared to MOCK (13332.31 ±3105.6) and CoStAR (1518.16 ±1586.7) transduced TILs. To further assess the migratory enhancement of these receptors, *in vivo* models represent a more comprehensive setting. OVCAR3 were selected to measure the trafficking of chemokine engineered HD T cells and TILs as they were identified to naturally produce the chemokines of interest. This cell line expresses folate receptor alpha (FOLR1) which is the target of the CoStAR (signal 2) and to provide signal 1 T cells were also transduced with the PRAME T-cell receptor (TCR). PRAME TCR+CoStAR T cells were able to kill the cell line and showed the highest cytokine production of interferon-γ (IFN-γ; 114553.02 ±63973.8 pg/mL) when compared to MOCK (122.51 ±38.8 pg/mL) and PRAME TCR T cells (28248.24 ±19767.4 pg/mL). Therefore, PRAME TCR+CoStAR T cells demonstrated functional activity, antigen recognition and cytotoxicity towards OVCAR3 *in vitro* and these findings are to be corroborated *in vivo*. The modification resulting in increased functionality and migratory capacity of the cells of interest will enable nomination of a candidate for intervention of TIL therapy in OC.

P0749

Humanised comparison of ‘universal’ lentiviral-integrated and CRISPR-mediated, virus-free CAR T cells

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Genomic insertion of chimeric antigen receptors (CARs) into T cells using nucleases – such as CRISPR-Cas9 – in combination with a homology-directed repair (HDR) template offers the possibility of site-specific CAR insertion as an alternative to existing viral-mediated CAR technologies. It has been suggested that CAR insertion into the T cell receptor alpha chain (*TRAC*) locus could result in improved function and persistence, given that transgene expression is placed

under the control of endogenous T cell receptor transcriptional machinery. Editing the *TRAC* locus can also ensure disruption of endogenous TCRαβ expression, reducing the risk of graft-versus-host disease and allowing cells to be used without matching. Similar objectives can be achieved by targeting insertion into other components of the multimeric TCRαβ/CD3 complex, including the CD3z chain, which additionally allows capture of endogenous CD3z signalling machinery into the CAR architecture. Direct comparisons of these virus-mediated and virus-free CAR T cells were undertaken using a CD20-specific CAR in established humanised models of B cell malignancy.

Virus-free CAR T cells were generated using SpCas9 ribonucleoprotein (RNP), in combination with synthetic, bacteria-free, linearised dsDNA template encoding anti-CD20 CAR and 300bp homology arms specific for either the *TRAC* locus or *CD3z* locus. Following delivery by electroporation into activated primary T cells, 'knock-in' efficiencies and simultaneous TCRαβ 'knock-out' ($TCR\alpha\beta^{-}CAR^{+}$) were quantified by flow cytometry after 7 days at 40.4% (mean, n = 12) for the *TRAC* site and 41.6% (mean, n = 4) at the *CD3z* locus. In comparison, lentiviral (LV) transduction yielded 66.5% (mean $TCR\alpha\beta^{-}CAR^{+}$, n = 4) in combination with *TRAC* knockout by electroporation of SpCas9 RNP. All products used for functional experiments were subjected to TCRαβ depletion by magnetic bead purification. As anticipated, 'knock-in' CAR-T cells exhibited less expansion with end of production yields lower than those achieved for LV-CAR products. Nonetheless, 'knock-in' CAR products performed similarly to LV-CAR in cytotoxicity assays and exhibited similar cytokine release profiles *in vitro*.

Next, CAR T cells were tested *in vivo* using a humanised tumour inhibition model comprising NOD/SCID/gamma (NSG) immunodeficient mice engrafted with luciferase-expressing Daudi B-cell lines. Serial imaging was performed weekly, and survival tracked over an eight-week period. Control animals survived to a median of 24 days (n = 8), and in keeping with leukaemic inhibition documented by imaging, CD3z-CAR20 animals (n = 10) survived to 31 days ($p < 0.0001$), whereas TRAC-CAR20 (n = 10) survived to 42 days ($p < 0.0001$) and majority of LV-CAR20 animals survived to the end of the experimental period, 56 days ($p < 0.005$).

In summary, we report the feasibility of generating donor-derived universal CAR-T cells using a virus-free platform and compliance-ready processes. While *in vitro* characterisation and functional studies were comparable and while all iterations inhibited leukaemia, longer survival was observed in humanised animals receiving LV-CAR T cells.

P0750

Enhancing CAR-T and CAR-NK cell therapy for solid tumors by targeting a proteolytically cleaved 8-amino acid peptide epitope

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Chimeric antigen receptor (CAR)-T cell therapy has shown remarkable success in hematologic malignancies but remains challenging for the treatment of solid tumors due to the limited availability of appropriate target antigens. Malignant cells possess multiple tumor escape mechanisms, including proteolytic cleavage of tumor antigens from the cell surface. To counteract this evasion strategy, we generated two CAR constructs based on an antibody targeting a surface-exposed, proteolytically cleaved 8-amino acid peptide remaining on the cellular membrane following protease cleavage of the native protein. The monoclonal antibody was

isolated from hybridoma cells and characterized for high specificity via ELISA and flow cytometric analysis of the epitope on cell lines modified to stably express the target peptide. The single-chain variable fragment (scFv) of this antibody was then used to construct second-generation CAR molecules for targeting tumor cells and primary T cells overexpressing the 8-amino acid peptide. CAR-T and CAR-Natural Killer (NK) cells targeting the processed peptide showed stable expression of the CAR molecules and exhibited robust proliferation. Furthermore, they showed CAR-mediated activation and specific lysis of various target cells in vitro expressing the 8-amino acid peptide but not an N-terminally elongated version of the processed peptide nor the native protein. Matrix metalloproteinases (MMPs) and sheddases like a disintegrin and metalloproteinase domain (ADAM) will be used in vitro to mimic the processing of the 8-amino acid peptide occurring in the tumor microenvironment. This new scFv targeting a short peptide superficially expressed and only common in malignant cells might contribute to more effective ways to avoid off-target and increase on-tumor effects of CAR-T and CAR-NK cell therapy.

P0752

Expansion of low-seed CAR T cells on a GMP-grade hollow-fiber bioreactor platform

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As autologous CAR T-cell therapies continue to evolve, cell quality and manufacturing time are critical parameters to reach their full potential.

Today, several platforms are available to achieve sufficient cell yields for therapeutic applications. Among them, the Quantum™ Cell Expansion System (Terumo Blood and Cell Technologies, Lakewood, CO), a functionally closed, hollow-fiber bioreactor platform, has been successfully used for automated expansion of a range of cell types and is used to significantly improve process efficiency relative to manual expansion. The next-generation Quantum Flex™ Cell Expansion System (Terumo Blood and Cell Technologies, Lakewood, CO) has been designed to build on Quantum's success and enhance usability and support in GMP-compliance by the addition of a server-based application. This Cell Processing Application (CPA™) can be used remotely to build customized protocols and monitor runs while documenting all changes, either in an audit trail or run report, of a fleet of up to 100 devices via network connection. The Quantum Flex system is used with a suite of non-DEHP disposables and operated with a disposable cell expansion set containing either a small- or standard-sized bioreactor.

In this study, CD19 CAR T cells were expanded in a small bioreactor on Quantum Flex using separate donors for each experiment. To assess the dynamic range of the platform, expansions were performed from different amounts of starting material (1 million to 15 million cells). Commercially available T cells were transduced with a lentivirus construct outside the bioreactor to generate anti-CD19 CAR T cells for this study. Cells were then loaded in the functionally closed and GMP-grade bioreactor and expanded for seven days, with viabilities remaining high throughout expansion. The cells in the bioreactor expanded 150- to 200-fold, achieving a maximum of 2.6 billion cells, compared to an average of 145-fold for transduced flask controls, in this study. Memory phenotypes and exhaustion markers at harvest were analyzed before final formulation and cryopreservation with the Finia™ Fill and Finish System (Terumo Blood and Cell Technologies, Lakewood, CO).

With this range of starting numbers of CAR T cells, the platform is relevant to adult, pediatric, and compassionate CAR T-cell expansion dosing. This study was a detailed investigation into the expansion of CAR T cells on Quantum Flex and fill-finish on Finia as part of the strategic collaboration between BioCentriq and Terumo Blood and Cell Technologies.

P0753

Riboswitch-regulated gene and cell therapy

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Controlled expression of delivered transgene is critical for both gene and cell therapies. Here, we report that by linking synthetic aptamer to an alternative splicing gene expression platform, we have created a robust, synthetic mammalian riboswitch cassette that regulates gene expression tightly and dynamically in response to small-molecule inducers. The splicing-based expression platform creates an “on” switch in the presence of the small molecule by sequestering a splice site of an alternative exon. Riboswitches that respond to these novel small-molecule inducers regulate transgene expression with high dynamic range in a dose-dependent manner. When delivered through an adeno-associated viral (AAV) vector to the liver or the muscle in mice, the engineered riboswitches reversibly regulate transgene expression via an orally delivered small-molecule inducer, providing precise control of transgene expression, with high dynamic range.

These switches were used to develop controlled gene and cell therapies. With these riboswitches and orally available small-molecule inducers, we were able to regulate hormones such as human growth hormone, growth factors such as erythropoietin (Epo), and therapeutic antibodies such as anti-HER2 antibodies to an efficacious level *in vivo*. RiboCAR-T cells with riboswitch-controlled chimeric antigen receptors (CARs) had more stem/memory-like phenotypes and exhibited superior anti-tumor activities against lymphoma when compared with conventional CAR-T cells that expressed constitutive CAR.

Thus, we have developed the first synthetic aptamer riboswitch that is capable of controlling therapeutic gene expression through orally available small-molecule inducers. This robust gene regulation system enables both temporal and spatial control of gene expression, providing not only improved efficacy but also a safety mechanism for both gene and cell therapies.

P0755

MHCII-TCR-engrafted CD4 T cells provide stronger and more durable T cell help in T-cell therapy of HBV infection than MHCI-TCR-engrafted CD4 T cells

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T-cell therapy represents a promising therapeutic approach to treat chronic hepatitis B virus (HBV) infection and HBV-associated hepatocellular carcinoma. To date, T cell therapies have focused on CD8 T cells transduced with MHCI-restricted TCRs as primary effector cells. However, CD4 T cells are known to play an essential role during the clearance of HBV infection. We, therefore, first investigated whether redirected TCR-engrafted CD4 T cells could help TCR-engrafted CD8 T cells. In co-cultures with HBV-expressing target cells, the addition of redirected HBV-specific CD4 T cells led to enhanced proliferation, cytokine release, and cytotoxicity on behalf of CD8 T cells. In a humanized mouse model infected with AAV-HBV, the transfer of TCR-engrafted HBV-specific T cells caused a significant decline of virological markers. It proved to be safe, with only limited and transient injury of the liver. While the transfer of CD4 or CD8 T cells alone already resulted in a drop of serum levels of HBeAg and HBsAg, the combination of CD8 and CD4 T cells was 2 to 3 logs more effective than monotherapy in controlling the viral antigen expression. This was accompanied by increased IFNg and TNFa secretion in CD8 T cells from the liver and spleen, as well as a lower viral load at the end of the experiment shown through qPCR of HBV DNA and HBV core antigen expression in the liver. In addition, single-cell RNA sequencing on day 8 revealed a population of highly activated cytotoxic CD8 T cells mainly present in the combination group. In clinical applications of T-cell therapy, CD8 and CD4 T cells are commonly transduced with the same receptor. We were, however, interested in investigating whether the MHC restriction of CD4 T cells mattered and therefore transferred CD4 T cells engrafted with an MHCII- or an MHCI-restricted TCR to serve as help for CD8 T cells. Indeed, we saw a more pronounced and more durable effect of T cell help *in vivo* when CD4 T cells were engrafted with an MCHII-restricted HBV-specific TCR. This was again shown by a faster and stronger drop in virological markers and significantly lower levels of HBV DNA and HBV core antigen expression in the liver at the endpoint of the experiment. Based on these data, we hypothesize not only a positive helper effect of CD4 T cells on CD8 cells, we believe that the MHC restriction of the transferred CD4 T cells matters, with MHCII-restricted CD4 T cells leading to a more pronounced and durable helper effect during T cell therapy of HBV infection.

P0756

Lentiviral vector-based polymeric nanoparticles for *in situ* gene delivery into T-cells for haematological malignancies & beyond

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With Abecma®, Carvykti®, Yescarta®, Kymriah®, Tecartus® and Breyanzi® marketed therapies for B cell hematological malignancies and more than 600 ongoing clinical trials, genetic modification of T cells with chimeric antigen receptors (CARs) recognizing surface antigens on tumor cells has emerged as a revolutionary therapeutic strategy in immuno-oncology. Despite impressive clinical benefits, the complex logistics required to manufacture *ex vivo* CAR-T cells from individual patients, the impact of such a process on the fitness and efficacy of engineered cells and the associated costs represent major hurdles to the widespread use of these therapies.

Alaya.bio is preclinical-stage biotechnology company developing *in situ* CARs based on nanoparticles consisting of lentiviral vectors lacking the VSV-G immunogenic protein and encoding anti-CD19 CARs coated with oligopeptide-modified poly(beta-amino ester)s biodegradable polymers.

Here we report on a microfluidics-based method that consistently manufactures polymeric nanoparticles able to reprogram *ex vivo* peripheral blood mononuclear cells to express a functional CAR. Importantly, stable delivery of the transgene is mediated without the need of CD3/CD28 activation and cytokines addition normally required with VSV-G pseudotyped LV for immune cells transduction. Fast and efficient nanoparticle-mediated gene editing of quiescent cells is achieved without labor-intensive processing and amplification which preserves reprogrammed cells from exhaustion, differentiation and does not jeopardize their functionality.

In vivo, the biodistribution of NPs has been investigated in immunocompetent and humanized mice after multiple intravenous administrations or infusions and showed a different profile compared to pseudotyped LVs. Long-term expression of GFP or CAR transgenes was detected in blood leukocytes up to 80 days post-treatment. The pronounced tropism for blood cells observed *in vivo* with our polymeric nanoparticles provides an obvious advantage for CAR T-cell therapy of blood malignancies.

Repeat administration was safe and well tolerated as no obvious sign of distress, body weight loss, hepatotoxicity, change in blood cell count or cytokine levels were reported. Preliminary efficacy data have shown in healthy Balb/c mice that repeat administration of anti-CD19 CAR encoding nanoparticles induced B cell depletion. In mice with A20-induced lymphoma, repeat treatment cycles showed promising anti-cancer efficacy and reinfusion at relapse was possible.

This technology shows great potential for further preclinical and clinical development of *in situ* universal CAR T-cell therapy. In addition, Alaya.bio's versatile platform offers the potential to target and reprogram a broad range of therapeutic cells not limited to the immuno-oncology field but also various genetic disorders.

P0758

Single-cell multi-omics analysis reveals differential lineage-specific vector copy number distribution in CAR-T cell product

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Cell and gene therapies are revolutionizing the treatment of genetic disorders, including cancer and inherited diseases. Among the most transformative approaches is Chimeric Antigen Receptor T-Cell (CAR-T) immunotherapy. To ensure the safety and efficacy of these modified cells, it is critical to accurately measure gene transfer efficiency and viral vector copy number (VCN) per cell. Carefully monitoring the intricate balance between administering a functional therapeutic product and avoiding excessive transgene copies that could lead to potential malignancies is essential for effective CAR-T therapy. Furthermore, since a "cell" is the basic functional unit of CAR-T therapy, it is prudent to measure these critical quality attributes (CQAs) in the context of single cells.

Conventional methods for quantifying VCN per transduced cell rely on the combination of multiple assays: e.g. FACS analysis to determine transduction efficiency, and qPCR or dPCR to determine sample VCN. However, these methods present limitations because not all transduced cells necessarily express the CAR transgene, and because qPCR or dPCR-based VCN readouts

report only population averages. Therefore these measurements suffer from low precision and accuracy, and cannot determine the distribution of VCN across cells.

In this study, we present a high-throughput, microfluidic, and amplicon-based single-cell protein + DNA multi-omic workflow designed to quantify transduction efficiency, vector copy number (VCN), and VCN distribution, as well as protein expression, across ~10,000 cells simultaneously. We validated the performance of the single-cell VCN assay using well-characterized clonal cell lines with known vector copy numbers as confirmed by orthogonal methods. The data obtained from the single-cell VCN assay showed a 99% correlation with dPCR assays. Cells were classified as transduced (VCN > 0) or non-transduced (VCN = 0) to calculate the percent transduction for each sample. Using mixtures of transduced and non-transduced cell lines, the % Transduction assay demonstrated specificity and sensitivity of over 98.8% and 99.2%, respectively. Notably, the VCN distribution of the sample was also reported, revealing the percentage of cells harboring specific VCN values.

We applied this single-cell protein + DNA multi-omic VCN workflow to analyze a bi-cistronic CD19 and CD22 CAR-T product. Our VCN analysis confirmed the distribution of VCN in the product closely adhered to the expected Poisson distribution throughout the expansion, suggesting a low risk of biased functional clonal expansion favoring cells with high VCN. Additionally, using a 45-plex protein assay, we quantitatively measured surface protein expression for lineage assignment. This revealed differential transduction percentages and VCN distribution patterns across cell lineages (e.g., CD4+ and CD8+ T cells), providing insights into factors potentially influencing treatment outcomes.

This single-cell multiomic transduction and VCN analysis provides unprecedented resolution and insight to assess the safety and efficacy of CAR-T therapy, as well as to aid in understanding the biology of cell therapies.

P0759

Depletion of the cytokine-induced SH-containing negative regulator of cytokine signalling CISH sensitizes induced innate T8 cells to IL-15 and enhances their T-to-NK transition

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Adaptive T cells with traces of innate characteristics like NKT or γδ T cells attracted the attention of translational researchers due to their combining the best of both immune compartments. Naturally sparse, which limits their broader clinical application, these unique effectors can be induced *ex vivo* by expanding naïve lymphocytes in the presence of interleukin 15. The transition of T cells to T/NK cell chimeras could be improved by deletion of the transcription factor BCL11B, that is critical for developing and maintaining T cell characteristics. The BCL11B-depleted, induced innate T8 cells (iiT8) upregulated further NK-specific functions compared to IL-15-only counterparts and became potent effectors with broad range of anti-tumour properties. However, preserving this advantageous phenotype and properties requires repeated stimulation

with IL-15 at concentrations that are not tolerated *in vivo*. Recently, it was shown that blocking the feedback mechanisms initiated by cytokine signalling may significantly reduce the dependence of T and NK cells on high levels of IL-15.

To investigate if the reprogramming of T to NK/T-cells can be further enhanced or achieved at lower, *in vivo* tolerable concentrations of IL-15, the gene encoding cytokine-inducible SH-containing protein (CISH), a negative regulator of signalling downstream of IL-15, was deleted individually or in combination with BCL11B. The comparison of the resulting T cell derivatives by immunophenotyping, transcription profiling and cytotoxicity assays confirmed the features of both types of single knockout cells reported so far. Excessive stimulation combined with increased sensitivity to apoptosis in CISH-depleted cells was not accompanied by improved T-to-NK transition, and the acquisition of NK cell features was observed in IL-15-supplemented BCL11B-deficient cells. Yet, cells missing both proteins revealed an intriguing combination of properties. The magnitude of NK-cell features rose significantly in these cells, manifested by a broader repertoire of activating NK receptors and soluble factors typical for innate cells. The adverse consequences of CISH inhibition, like stress sensitivity, induction of checkpoint molecules, and other immunosuppressive mechanisms, could be abolished by simultaneous targeting of BCL11B locus. These cells outperformed in three different cytotoxicity assays, being more efficient than iiT8 cells in the spontaneous killing of transformed cells and when assisted by a therapeutic antibody. They also appeared to be very effective carriers of chimeric antigen receptors (CAR) and maintain their activity at significantly lower levels of IL-15.

Collectively, although the loss of CISH alone did not improve IL-15-induced acquisition of NK features in T8 lymphocytes, the simultaneous depletion of BCL11B and CISH produced iiT8 putatively capable of executing their functions at IL-15 concentrations that prevent *in vivo* toxicity.

P0760

Microfluidic squeezing is a suitable mechanical method for CAR-T cell engineering

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The field of immunotherapy is witnessing a revolutionary shift through immune cell engineering, involving the genetic modification of T cells, natural killer cells, macrophages and other cell types. Chimeric antigen receptor (CAR) T-cell therapy, in particular, has shown remarkable efficacy in B cell malignancies and is showing promising results in solid tumours. Viral vectors remain the method of choice for T cell modification, however, they present important limitations, such as high cost and stringent regulatory demands due to their potential mutagenetic character. To overcome these challenges, there is a growing interest in robust non-viral approaches, such as mRNA transfection. Here, we demonstrate efficient and robust intracellular mRNA delivery to primary human T cells through microfluidic squeezing. A major advantage of this approach is its simplicity, as it only requires a microfluidic chip and a pressure regulation system, and its high throughput processing of up to 10 million cells per second. This approach enabled the engineering of functional CAR T cells, demonstrating its potential for future applications in mRNA-based cellular engineering research and clinical use.

P0761

Addressing Cellular Resource Competition to Enhance Dual Transgene Expression in CAR-T Cells

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A key challenge for Chimeric Antigen Receptor (CAR) T cell therapy is tumor escape mediated by antigen loss. One strategy to mitigate this limitation is to develop CAR-T cells targeting multiple antigens, but the optimal method for creating such multi-targeting CAR-T cells remains undefined. We aimed to develop dual-targeting CAR-T cells by co-transducing T cells with two lentiviral vectors, each encoding a different CAR. This strategy results in three distinct CAR populations: one expressing both CARs, and two expressing either CAR alone. Considering that cellular resources are limited, we hypothesized that co-expression of two CARs in the same T cell could compete for the transcriptional and translational machinery, potentially impairing the expression of one or both CARs on the T cell surface and causing an imbalance in CAR-T cell populations.

To investigate this hypothesis, we co-transduced T cells with various combinations of CAR constructs, each driven by the same promoter and with similar construct sizes, including ARI-0001 targeting CD19, ARI-0002 targeting BCMA (both 4-1BB-based CARs developed at Hospital Clínic de Barcelona-IDIBAPS), and CD28-based CARs targeting HER2 or mesothelin. We found that co-transduction generally resulted in lower expression levels of one or both CARs compared to single CAR transductions. This impaired CAR expression was more pronounced at higher multiplicities of infection (MOI), indicating increased competition for limited cellular resources as saturation approached.

To identify potential rate-limiting steps in this competition, we analyzed CAR expression at the DNA, RNA, and protein levels. CAR integration remained consistent whether transduced alone or with another CAR; however, RNA levels were lower in co-transductions, indicating that the transcriptional machinery is particularly sensitive to the burden of multiple transgenes. Testing various codon-optimized CAR versions and sequentially transducing the two lentiviral vectors did not mitigate the decreased CAR expression after co-transduction.

Ultimately, we demonstrated that using different MOIs for the co-transduced CARs can effectively generate dual-targeting CAR T-cells with balanced populations of each CAR when using the co-transduction technique.

These preclinical findings highlight the critical importance of carefully controlling lentiviral quantities in manufacturing T cells or other cells harboring multiple transgenes, to prevent resource saturation and maintain intact endogenous expression.

P0762

Development of adoptive T cell therapy targeting shared immunogenic tumor antigens in sarcoma patients

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Adoptive T cell therapy with engineered T cells to express a tumor specific T cell receptor (TCR) or Chimeric Antigen Receptor (CAR) is a successful approach for the treatment of cancer patients. However, the paucity of “universal”, tumor-specific antigens represents one of the major bottlenecks for wider implementation of cell therapy in clinical practice.

Recently, our lab developed a pipeline to successfully identify immunogenic tumor-derived antigens and shared among cancer patients. Specifically, our group proved that *Salmonella* infection of tumor cells exacerbates endoplasmic reticulum (ER) stress and induces the opening of hemichannels forming gap junctions, thus causing the release of tumor-derived immunogenic peptides. Notably, only vaccines prepared with the most immunogenic peptides isolated from the secretome of *Salmonella*-infected primary tumor cells, but not from the one of non-tumorigenic healthy cells, foster antitumor responses in melanoma mouse models and in dogs affected by osteosarcoma (Melacarne et al., *Cell Reports* 2021; Marconato, Tiraboschi et al., *Cancers* 2023).

Here, we performed mass spectrometry and peptidomic analysis of 28 primary sarcoma secretomes. We identified multiple peptides upregulated in *Salmonella*-infected primary tumor cell lines compared to not infected cells. Interestingly, we identified a group of peptides derived from tumor relevant antigens and shared among a large fraction of analyzed sarcoma patients. Furthermore, bioinformatic analysis identified predicted HLA-restrictions of the relevant peptides.

Ongoing in vitro experiments aim at validating immunogenicity and HLA-restriction of the identified peptides. Next, peptide-specific TCR will be identified using healthy donor and patient derived T cells.

Our approach will ultimately lead to the development of off-the-shelf cell products targeting shared tumor antigens.

P0763

Creating Universal Anti-CD19 CAR T Cells with a Memory Phenotype

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Chimeric antigen receptor-expressing T cells (CAR T cells) have revolutionized the treatment of cancer, particularly in B cell malignancies. Despite their success, CAR T cell therapy faces significant limitations due to the reliance on autologous T cells, which can lead to increased costs, variable efficacy, and adverse effects related to cell phenotype. To overcome these challenges, we have developed a novel strategy to generate universal and safe anti-CD19 CAR T cells characterized by a defined memory phenotype.

Our approach utilizes CRISPR/Cas9 technology for the precise elimination of the B2M and TRAC genes. This genetic modification reduces the risk of both graft-versus-host disease (GVHD) and host-versus-graft rejection. Additionally, we select for less differentiated T cells to enhance the stability and persistence of the universal CAR T cells, thereby improving their therapeutic potential.

To ensure the safety and efficacy of our CAR T cells, we conducted comprehensive transcriptomic and genomic analyses. These evaluations provided robust evidence of successful gene knockout and confirmed the absence of unintended off-target effects on gene expression and overall transcriptome integrity. Our findings underscore the importance of thorough genomic and transcriptomic assessments in validating the safety of genome-edited cell therapies.

In vitro studies demonstrated that our memory universal CAR T cells exhibit potent cytotoxic activity against tumor cells while maintaining a more controlled cytokine secretion profile. This balance is crucial for reducing potential side effects associated with excessive cytokine release, a common complication in CAR T cell therapies.

In conclusion, we have established an effective and scalable pipeline for the production of safe universal CAR T cells with a favorable memory phenotype. This advancement holds promise for enhancing the accessibility, efficacy, and safety of CAR T cell therapies, potentially extending their benefits to a broader patient population. Our work underscores the potential of CRISPR/Cas9 technology in developing next-generation cell therapies and highlights the importance of rigorous safety evaluations in the context of genome editing.

P0764

Enhancing CAR T cell therapy for solid tumors using Prussian Blue nanoparticle-based photothermal therapy (PBNP-PTT)

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High-risk neuroblastoma is an extracranial childhood cancer that despite advances, poses treatment challenges due to its aggressive and heterogeneous nature. The five-year survival rate

for high-risk patients remains at 50%, and this grim outlook provides the impetus to improve therapeutic strategies against neuroblastoma. Chimeric antigen receptor (CAR) T cells is a highly promising treatment option as it involves the adoptive transfer of genetically modified T cells that can recognize and kill cancer cells in a targeted manner. While CAR T cells have generated unprecedented response rates in B cell malignancies, its efficacy has been limited in solid tumors due to antigen heterogeneity and suppressive tumor microenvironment (TME). Advances in nanomaterials for photothermal therapy (PTT) may potentially overcome these challenges and in combination with CAR T cells, serve as a curative multimodal therapy. PTT employs the use of nanomaterials to absorb near-infrared light and convert it to heat in a localized and minimally invasive manner. Our group has demonstrated that Prussian blue nanoparticle (PBNP)-based PTT ablates tumors, promotes immune cell recognition and engagement by damage associated molecular patterns, and enhances the impact of immunotherapies, including for treating neuroblastoma (PMID: 29665282, 27826115). Given these observations, we hypothesize that PBNP-PTT can prime tumor cells for augmented targeting and killing by CAR T cell as a combination therapy for neuroblastoma.

We first determined whether PTT modulated the expression of B7H3, a prognostically-significant biomarker and a CAR T cell therapeutic target for neuroblastoma. We validated that cell death correlates with increasing thermal doses in PTT treated neuroblastoma cell lines SH-SY5Y and LAN-1, while high basal B7H3 expression is sustained throughout treatment. We then generated B7H3-targeting CAR T cells from healthy donors (n=3). To evaluate if the cytotoxicity of CAR T cells against neuroblastoma is improved with PTT, we performed PTT on human neuroblastoma lines SH-SY5Y and LAN-1 and subsequently cocultured them with either non-transduced or B7H3-targeting CAR T cells. Functional analyses evaluating cytotoxicity and cytokine secretion indicate that antitumor activity is improved with the combination of B7H3 CAR T cells and PTT versus either treatment alone. Currently, we are evaluating the combination of B7H3 CAR T cells with PTT in a syngeneic murine neuroblastoma model and its capability to remodel the TME for enhanced immunotherapy efficacy. Overall, our findings support the proposed use of PTT for enhancing CAR T cell therapy as a novel treatment strategy for neuroblastoma.

P0765

Development of activation-inducible promoters for the improvement of CAR-T therapy

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Tumour immunotherapy aims to enhance the immune system to eliminate tumour cells specifically. One type of immunotherapy that is achieving the best results is based on the genetic modification of T cells to generate CAR-T ("Chimeric Antigen Receptors"). Specific anti-CD19 CAR-T cells have achieved unprecedented successes in patients with refractory B-cell malignancies. However, there is still a lot of room for improvement due to the strong side effects, such as Cytokine Release Syndrome and neurotoxicity due to overactivation of CAR-T cells where or when they should not. The ability to turn transgene expression on and off at exactly the right time can therefore significantly improve the safety of this immunotherapy. In this line, different

activation-inducible promoters based on the consensus binding sequence of the transcription factor NFAT (named NFATsyn) have been designed (Zimmermann, K. *et al*. 2020). However, these promoters showed expression in the absence of activation and prolonged expression over time. Optimal control of gene expression requires low background activity in the absence of induction and high activity in the presence of the effector.

In this study we propose to generate more effective CAR-T cells with potentially lower side effects by regulating the expression of bioactive molecules such as IL12 or IL18 in short periods of time through the use of inducible promoters that will start the transcription only after the activation of the T cell. Three chimeric promoters have been designed based on genes involved in the activation of T cells incorporating different combinations of transcription factor binding sites. These promoters were inserted into a lentiviral backbone to express GFP as a reporter gene to track the promoters activity. Primary human T cells were transduced with these GFP-expressing lentiviral vectors through inducible promoters and GFP expression after activation via CD3/CD28 was analysed. We have seen that our promoters increase GFP expression reaching a peak at 8 hours and a minimum at 24 hours. After 24 hours of activation, GFP expression gradually increases until 72 hours when it decreases again. Although our promoters increase GFP expression 5-fold upon activation, they exhibit high basal expression in the absence of activation. Due to this, a second generation of the best candidate has been developed to decrease leaking. This modification has been performed by eliminating fragments of enhancer sequences or by adding a repressor sequence of an endogenous gene involved in the activation of CAR-T cells obtained through RNAseq.

P0766

Ligand-Induced assembly of antibody variable fragments for gene regulation and cancer immunotherapy

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Gene-, protein-, and cell-based therapies are increasingly being applied to treat a broad spectrum of diseases, from genetic disorders to cancer. Despite significant clinical successes over the past decade, which have led to the approval of several such therapies, safety concerns still impede their wider adoption. Uncontrolled activation of therapeutic genes or cells can cause serious side effects in patients, which could be mitigated through external pharmacological control of therapeutics. Small molecule-inducible regulation has been implemented to enhance the safety of biological therapeutics by allowing precise and tunable modulation of their expression, stability, and activity.

Chemically induced dimerization (CID) systems rely on small molecules to trigger the association of two protein domains that interact only in the presence of the inducer molecule. These tools have been utilized in numerous research applications, including the development of genetic circuits. However, the clinical implementation of CIDs is limited due to the unfavorable characteristics of several small molecules used and the non-human origin of protein dimerization components, which can raise immunogenic responses in engineered cells. Given their favorable characteristics and programmability, antibody variable regions could represent ideal building

blocks for constructing CIDs with therapeutic utility, inducible by clinically approved molecules with established safety profiles.

We have designed a chemical molecular tool for the conditional regulation of protein expression and biological activity with therapeutic utility. The platform consists of human-derived antibody variable fragments (Fvs) that bind small molecules. We demonstrate that individually expressed variable heavy and light chains dimerize only in the presence of the selected ligand. Ligand-responsive Fvs were utilized to control both exogenous and endogenous gene expression in mammalian cells via CRISPRa, demonstrated using nicotine and estradiol. Additionally, the fluorescein-responsive Fv-CID system was applied for highly efficient conditional tumor cell killing by engineering inducible chimeric antigen receptors (CARs). We also showcase the fluorescein-regulated functional bispecific T cell engager (BiTE) that can effectively control the activity of T cells to eliminate tumor cells both in vitro and in vivo.

This study establishes a customizable platform for generating CIDs from antibody variable fragments that bind small molecules. This technology provides a method for the pharmacological control of gene-, protein-, and cell-based therapeutics, thus significantly enhancing their safety. The ability to precisely regulate therapeutic activity in response to clinically approved small molecules opens new avenues for safer and more effective treatments, addressing current limitations in the field and potentially expanding the clinical applications of advanced biological therapies.

P0767

One-step generation of allogeneic CAR-T cells by simultaneous multiplex knockout and site-specific transgene integration with the Pin-point™ base editing platform

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As our understanding of immune function regulators and the effects of the tumour microenvironment on immune cells improves, increasingly more complex cell engineering is being employed to improve the efficacy of cellular immunotherapies. We have developed the RNA aptamer-mediated Pin-point base editing platform with the aim to facilitate this complex cell therapy engineering in a safer manner than conventional nuclease-based technologies. The Pin-point base editing system is a modular technology where the CRISPR-Cas and the deaminase modules are delivered to the target cells as individual components. The assembly of the base editing machinery at the target locus relies on the interaction between an aptamer binding protein fused to the deaminase and an RNA aptamer located on the gRNA. The modularity and aptamer-dependent nature of the technology supports high flexibility in the customization of each individual component to address specific editing needs and enables complex genetic modifications. In this example, by combining aptamer-containing and aptamer-less gRNAs, we generated functional engineered CAR-T cells via simultaneous knockout of multiple targets by base editing alongside targeted chimeric antigen receptor (CAR) insertion at the endogenous TRAC locus. We used aptamer-less gRNAs to direct the nickase activity of the Cas enzyme to both DNA strands of the integration locus and stimulate homologous dependent repair (HDR) but

avoiding the recruitment of the deaminase; whilst in the same transfection, aptamer-containing gRNAs recruit the deaminase to the other target sites for knockout generation by base editing. With this approach, site-specific knock-in and multiplex gene knockout are achieved within a single intervention and without the requirement to deliver additional sequence-targeting components, the introduction of multiple nuclease species, or more convoluted sequential editing strategies. We demonstrated high base editing efficiency and confirmed the safety of this approach by assessing the editing purity at all target sites, and carefully characterising the occurrence of DNA and RNA off targets, as well as genomic structural variants. The modularity and aptamer-dependent nature of the Pin-point base editing technology opens the possibility of specifically optimizing editing for each site in a different way and of combining multiple effectors to achieve advanced editing outcomes, broadening the applicability of this editing approach across oncology, autoimmunity, and the treatment of rare disease.

P0768

In vitro model of Cytokine Release Syndrome linked to treatment with CAR-T cells

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Chimeric antigen receptor (CAR)-T cell therapy uses genetically edited T cells to target specific antigens on the surface of disease-inducing cells, leading to their elimination. Due to its precise approach, high efficient output and ease of cell sourcing, it has been increasingly explored as an attractive tool to treat cancer for the last decade, including non-oncologic indications recently. Great clinical success in treating B-cell malignancies has led to a widespread investment on this therapy, resulting in a significant increase in the number of patients treated with CAR-T cells in recent years. Despite the promising efficacy data generated in these trials, key safety concerns have also been highlighted, including the highly prevalent and potentially life-threatening cytokine release syndrome (CRS).

CRS is an intense cytokine-mediated inflammatory response originating not only from the cytotoxic activity of the activated T cells but also from the host immune cells responding to the activation and from the dying target cells themselves. Whereas CRS is frequently a mild and self-limiting event that can be easily managed clinically, it can progress in severity to high fevers, hemodynamic compromise and severe hypoxic processes that can, in extreme cases, lead to death of the patient. Despite it being well monitored in the clinic, current capacity for its preclinical prediction is still poor. Furthermore, this is a risk that varies depending on the characteristics of the CAR-T cell product itself as well as on the specific microenvironment found in each particular disease context. As such, when designing a new CAR-T cell product, it becomes crucial to be able to predict accurately its specific potential to cause high-grade CRS prior to human clinical trials, especially for non-oncologic indications, where the acceptable level of safety risk must be necessarily much lower. However, there are very few preclinical models capable of assessing the risk of CRS confidently.

In this work, we established a tri-culture *in vitro* cell model representative of the target cells / host immune system / CAR-T cell interactions found *in vivo*. It is a tool that can be used not only for preclinical assessment of the propensity of any CAR-T cell product to provoke the generation of an intense CRS event but also to understand its underlying mechanism. In this model, B cells were used as target cells and T cells were edited with a BCMA-specific CAR. To represent the inflammatory response of the host immune system, the model was further complexified by adding

monocytes, macrophages and/or dendritic cells to the co-culture. Levels of up to 50 inflammatory cytokines (including IFNγ, IL-1β, IL-6 and TNF-α) were measured in the supernatant and/or cell lysates to ascertain contribution of each cell type for the overall levels of CRS observed. Cell viability and phenotype were also assessed for each cell type via flow cytometry analysis to better understand the intercellular dynamics underlining the CRS cascade observed.

P0769

Engineering T cells with newly designed synthetic microRNAs enhance lymphocytes functionality and reduce exhaustion

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Despite the success in treatment of liquid malignancies, CAR-T treatment for solid tumours still face several hurdles. Immunologists and bioengineers are thus working synergistically to address challenges such as i) low persistence and functionality of T cells within the tumor microenvironment(TME); ii) the life-threatening side effects caused by an excess of CAR-T activation and cytokine release syndrome (CRS); iii) tumor antigen escape. Specifically, in our work we tried to address the first two problems. First, loss of functionality of T cells in the TME is caused by chronic antigen exposure, a condition better known as exhaustion. This represents a dysfunctional state in which CD8+ T cells lose their avidity, promoted by tumor and inhibitory immune cells. Second, life-threatening side effect are caused by uncontrollable activity of CAR-T cells, which are confined in an ON configuration that do not guarantee controllable cytotoxic response. In this picture, synthetic biology is becoming a new key player in engineering cell-based immunotherapies, with the aim of creating spatial-temporal genetic circuits which sense environment inputs and trigger controlled outputs.

Current methods targeting exhaustion-driver genes focus on the genes knock out which is not ideal to keep the physiological function of the T cells. We reasoned that by confining the expression of target genes within the T cell effector-associated expression windows, would provide a better strategy to maintain the functionality of T cells.

Here, we engineer T cells with novel post-transcriptional regulators, synthetic microRNAs (SyNmiRNA) to augment the efficacy of the immunotherapy. In general, our targets are cytoplasmic proteins downstream the TCR signaling and nuclear proteins whose upregulation is associated to exhaustion or to the halted T cell functionality. We generated different SyNmiRNA by including the DNA-encoded shRNA embedded in pri-miRNA-155 scaffold. As we are filing several IPs, we cannot disclose all the targets of the SyNmiRNA, we will thus specifically focus here on the SyNmiRNA targenting TOX and CISH. TOX is a master regulator of exhaustion and controls the expression of different immune checkpoint receptors, whereas CISH inhibits PLCg downstream TCR signalling pathway.

We tested first the silencing ability of SyNmiRNA in a model cell line. Next we validated the exhaustion-mitigation and the improved functionality of T cells by delivering them in primary CD8+ T cells. We observed that the downregulation of CISH gene results in higher Granzyme B secretion whereas downregulation of TOX reduced exhaustion markers in three healthy donors.We are currently coupling the SyNmiRNA to synthetic exhaustion sensors. The synthetic

sensors respond to transcription factors progressively upregulating in T cells that are prompted towards the exhaustion state. This will enable the specific and reversible activation of the SyNmiRNA in a cellular-state specific fashion.

In summary, we developed a versatile strategy to overcome T cell exhaustion that operates locally in the TME and induces a genetic switch from ON to OFF according to the external stimuli. Our constructs could represent the starting point for the development of novel immunotherapy that could be used to potentially overcome current limitation is the treatment of solid tumor.

P0770

Production of lentiviral vectors for the generation of Car-T Cells in an integrated manufacturing process in stirred-tank bioreactors

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Adoptive cell therapies using Chimeric Antigen Receptor (CAR)-T cells have shown great potential for treating haematological malignancies. However, such success has not been observed in solid tumours, in part due to suboptimal CAR-T cell persistence in vivo given the harsh microenvironment of the tumour and the lack of defined target antigens. Additionally, T cells' ability to engraft is related to their differentiation state as an increase in naïve/central memory cells has been linked to improved clinical outcomes. Other subject about CAR-T manufacturing process is that despite the recent advances in developing alternative non-viral approaches to engineer T cells, lentiviral vectors (LV) are still the main tool used in pre-clinical and clinical studies to modify these T cells. The production of LV is also an important part CAR-T production, imposing high costs to the final product. Given the vast potential of CAR-T therapies and the fact that most of the diagnosed cancers are primarily solid tumours, it is expected that the development of scalable and more efficient CAR-T cell manufacturing processes will contribute to decrease the inherent costs associated to CAR-T manufacture, ultimately benefiting the patients.

In this work, as a proof-of-concept of an integrated and scalable process to manufacture CAR-T cells targeting a solid tumour in stirred-tank bioreactor, we developed a microbead-based system (microbeads functionalized with anti-CD3/CD28 epitopes) that tightly controls initiation/termination of T cell activation through the establishment of differential agitation rates in small-scale STB (ambr15®). To produce a CAR-T product targeting solid tumours we generated a second and third generation CAR against the human epidermal growth factor receptor 2 (HER2). HER2 overexpression is detected in several types of cancer such as breast, gastric and ovarian cancers.

The CARs were developed by replacing the anti-CD19 targeting regions of a CAR previously generated in our laboratory by an anti-HER2 sequence from the Trastuzumab (a monoclonal antibody that binds to domain IV of the extracellular segment of the HER2 receptor). To better assess the ability of the manufactured CAR-T cells to penetrate a solid tumour model developed in vitro, we developed CAR expression cassettes that co-express a green fluorescent protein (GFP), through an internal ribosome entry site (IRES).

To validate the optimal conditions for the CAR-T generation, we evaluated LV transduction efficiency and its correlation with distinct timing of LV transduction following T cell activation. Additionally, the impact of several transducer enhancers in transduction efficiency and cell

toxicity were assessed. The specificity and function of the manufactured CAR-T cells targeting HER2-expressing breast cancer cell lines were evaluated in killing assays.

Overall, our work could contribute to develop scalable manufacturing processes towards the generation of CAR-T cells by streamlining the LV transduction step, amenable to be performed in stirred-tank bioreactors.

P0771

Establishing a clinical TCR candidate library through systematic and scalable cell-based platform

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TCR-modified T-cell therapies (TCR-T) exploit the property of TCRs to selectively recognise peptide-HLA (pHLA) complexes expressed on tumour cells. By targeting antigens that are presented on the tumour cell surface through pHLA complexes, TCR-T therapy renders a vast and previously untapped epitope target space accessible, including tumour-restricted epitopes of intracellular antigens. To deploy effective TCR-Ts against the large collection of tumour-derived epitopes presented across a broad range of HLA alleles, a comprehensive library of clinically actionable TCRs is desirable. However, the identification of potent and sensitive TCRs as well as their in-depth safety characterization at scale has been challenging.

Here, we describe a scalable and systematized human cell-based end-to-end platform for the identification of novel target pHLAs as well as identification and validation of cognate TCRs and subsequent safety profiling. Our platform leverages an integrated molecular genetics and cellular engineering “plug-n-play” workflow for the rapid and parallel production of either HLA-expressing mono-allelic engineered antigen-presenting cells (eAPCs) or engineered TCR-presenting cells (eTPCs).

In an unbiased manner, tumour-restricted pHLAs were identified and validated using target antigen-expressing eAPCs by mass spectrometry. To identify cognate TCRs, naïve human $CD8^+$ T cells were stimulated by eAPCs and candidates of paired TCRα/β sequences were captured using custom-built pHLA multimer reagents.

To comprehensively measure and rank TCR sensitivity and specificity towards its target pHLA, we developed a high-throughput method using eTPCs screened across eAPCs and orthogonal cancer cell lines. In these coculture assays, activation of TCR signalling-linked fluorescent reporters enabled the robust and reliable classification of candidate TCRs.

To proactively mitigate clinical risk, the cross-reactivity and alloreactivity profiles of each candidate TCR were comprehensively characterized. To identify potential cross-reactivity, the physiochemical tolerances of each candidate TCR were determined using a panel of eAPCs expressing saturating point mutations across the target peptide. Subsequently, candidate TCR cross-reactivity was assessed in coculture with eAPCs encoding minigenes of all genome-encoded potential cross-reactive peptides. These processes have reliably identified known cross-reactive TCRs and de-risked multiple novel TCRs. To assess potential allo-reactivity, candidate TCR reactivity was tested against a panel of 86 common HLA mono-allelic eAPC lines.

Finally, to overcome potential limitations of the natural TCR repertoire, we have developed a synthetic TCR directed evolution workflow to up-cycle sub-optimal TCR frameworks. This workflow allowed us to successfully remove unwanted cross-reactivity while maintaining on-target reactivity, and to improve a low sensitivity TCR to a clinical candidate equivalent.

Importantly, our platform highlights that multiple pHLA combinations can be derived from a single tumour-restricted sequence. Moreover, the sensitivity of our approaches permits key insights into the immunogenicity of a specific pHLA target based on the frequency of target-reactive TCRs captured from the naïve T cell repertoire.

In conclusion, utilizing this precision cell-based analytics and selection platform, we have identified clinically actionable TCRs targeting both neoantigens and shared tumour antigens across multiple HLA alleles. This platform is constructed specifically to deliver pHLA targets and cognate therapeutic TCRs at a scale that can deliver TCR-T libraries for personalized therapy.

P0772

Development of cell-targeted lipid nanoparticle for *in vivo* genetic medicines targeting hepatocytes, T-cells, and hematopoietic stem cells

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Realizing the full potential of genetic medicines requires redosable, non-immunogenic, in vivo delivery systems that provide cell selective biodistribution to multiple tissues beyond the liver, and that can be manufactured at scale. To this end, Generation Bio has developed cell-targeted lipid nanoparticles (ctLNP) for the in vivo delivery of genetic medicines to hepatocytes, and also to T-cells and hematopoietic stem cells.

Our ctLNP is comprised of three primary components: a stealth LNP capable of carrying both DNA and RNA cargos, an optimized bioconjugation linker, and a modular targeting ligand that directs receptor-selective biodistribution of the ctLNP to the intended cell population. The stealth LNP exhibits minimal protein corona formation upon exposure to serum proteins and retains endosomal escape properties, allowing for efficient cargo delivery when directed to cells of interest with a targeting ligand. In mice and NHP, the untargeted stealth LNP exhibits reduced off-target clearance by liver and spleen and an extended residence time in the blood, properties that are required for potent, selective targeted delivery. Cell-targeting is achieved through site-specific bioconjugation of high affinity, cell-selective ligands to the surface of the stealth LNP. Optimization of the ligand format, bioconjugation linker chemistry, linker length, ligand density and conjugation process improved stability and potency of the ctLNP.

Our demonstration of cell-type-specific targeting began in the liver with the asialoglycoprotein receptor (ASGPR), which has been clinically validated as a target for delivery of small interfering RNA and antisense oligonucleotides to hepatocytes. Most commonly, ASGPR is targeted using multivalent GalNAc ligands which have high affinity for the multimeric ASGPR complex. ctLNPs

conjugated with an ASGPR-selective VHH ligand provided higher hepatocyte delivery of DNA encoding luciferase *in vitro* and in mice than ctLNPs containing a GalNAc targeting ligand.

We next demonstrated targeting of the ctLNP to T-cells. We identified highly specific T-cell targeting ligands, further optimized ligand conjugation and demonstrated dose-dependent delivery of mRNA encoding GFP to human T-cells *in vitro* and in humanized mice with minimal off-target delivery to myeloid & B-cell populations and low distribution to off-target tissues like liver and spleen. These ctLNPs have subsequently been used to drive high levels of functional CAR expression *in vitro* and robust cell-surface CAR expression on T-cells in humanized mice.

The highly efficient workflows developed to identify T-cell ligands are now being utilized to develop an HSC-targeted ctLNP for *in vivo* delivery of RNA-based editors to increase fetal hemoglobin expression for the treatment of sickle cell disease and beta-thalassemia. We have identified targeting ligands for multiple HSC receptors, demonstrated efficient bioconjugation to stealth LNPs and generated highly potent conjugates that transfect human HSPCs.

P0773

Accelerating clinical translation of advanced therapeutic medicinal products: Leveraging GMP simulation facilities and biofabrication expertise

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Advanced therapy medicinal products (ATMPs), including gene therapies, (engineered) T cell therapies and tissue engineering products, are emerging as crucial elements for the patients with limited treatment options. ATMPs have demonstrated significant therapeutic benefits in the field of oncology as well as regenerative medicine. However, the successful development of an ATMP requires a specialized technology translation from a research-grade product into a good manufacturing practice (GMP)-compliant production process. The intrinsic heterogenicity and complexity of ATMPs, the lack of standardization across manufacturing and QC methods, and the insufficiency of translational expertise of academic researchers are the main obstacles hindering rapid development of ATMPs. Addressing these challenges, the concept of a GMP simulation unit has emerged as a promising solution for bridging the gap between innovative ATMP research and its clinical translation. By closely mimicking GMP manufacturing environments, GMP simulation units not only enable early identification of challenges and optimization of production workflows, but also reduces potential costs related to late-stage product development and production failures, ultimately advancing the development and deployment of these innovative treatments. In light of this, the Innovation Center of Advanced Therapies (ICAT), embedded within the University Medical Center (UMC) Utrecht has established a unique “academic ATMP development” framework. This set-up consists of three main components: (i) a Biofabrication Pilot Facility that advances tissue engineering and regenerative medicine through cutting-edge 3D bioprinting technologies (ii) an academic GMP Simulation Facility dedicated to the acceleration of ATMP clinical translation, and (iii) a GMP Production Facility that provides manufacturing of ATMPs in compliance with relevant guidelines under a GMP manufacturer license.. ICAT’s core team comprises not only experts in viral vector development, cell and gene therapy production, assay development, biofabrication and tissue engineering, but also specialists in GMP, quality and

regulatory affair. This remarkable transdisciplinary collaboration is designed to overcome the barriers in translating ATMPs from the bench-to-bedside. The integration of a GMP simulation infrastructure, GMP production facility, and biofabrication capabilities, combined with early-stage collaboration among multidisciplinary experts in an academic medical setting, aims to streamline the development and clinical application of ATMPs. By doing so, ICAT is positioned to make significant contributions to the advancement of personalized and regenerative medicine, ultimately improving patient outcomes and expanding treatment options for conditions with previously limited solutions.

P0774

Investigating T-cell expansion in bioreactors: a comparative approach with shaken flask and static culture methods

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The evolution of gene and cell therapy is contingent upon the development of robust and scalable T-cell expansion protocols. This study aims to explore the potential of bioreactor systems, employing both fed-batch and perfusion techniques, as a means to enhance T-cell proliferation in comparison to traditional shaken shake flask controls and static cultures such as T-flasks, bags and G-Rex devices. Preliminary data suggest that perfusion bioreactors may offer a promising alternative, potentially leading to increased T-cell yields in a single vessel, which is a critical factor for the success of gene and cell therapies.

A key focus of the research is to assess whether the dynamic conditions within bioreactors impact the phenotype of the expanded T-cells, with an emphasis on maintaining the integrity of cellular characteristics crucial for therapeutic applications. The study also intends to examine the expansion efficiency of various T-cell subsets, including CD4+/CD8+ at a 1:1 ratio, CD4+ only, and CD8+ only, within each bioreactor setup.

The rationale for comparing bioreactor systems with shaken shake flasks lies in the latter's dynamic culture environment, which may more closely resemble the fluid dynamics encountered in bioreactors than static cultures do. This comparison is essential to understand the potential benefits of bioreactors over static culture methods, which are often constrained by scalability, in-process control and suboptimal gas exchange.

The anticipated outcomes of this study could highlight the advantages of bioreactor technology for gene and cell therapy applications, particularly in terms of scalability and the ability to maintain cell quality during expansion. While the results are pending, the implications of this research could be significant, offering insights into the optimization of T-cell production processes and contributing to the advancement of therapeutic strategies.

P0775

Development of a novel iCasp9 kill switch for safer cell therapies

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Several Chimeric Antigen Receptor (CAR)-T cell therapies are approved for haematological tumours while many are in development and trials for solid tumours. However, due to the risk of lymphoproliferation, cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS), it is important to engineer cell therapies with so-called suicide or kill switches that can be activated in such instances. Here, we describe SIM-iCasp9, a novel caspase-9-based kill switch activated via the orally available, clinically approved, small molecule simeprevir. This feature provides significant advantages over existing iCasp9 kill switches that are activated by small molecules that have not yet been approved. We have demonstrated that activation of the SIM-iCasp9 kill switch can provide effective killing of several cell types *in vitro* including cell lines, ES cells and immune cells, via rapid induction of apoptosis. Furthermore, when SIM-iCasp9-expressing tumour cells are implanted into mice, administration of a single dose of simeprevir led to complete tumour regression. Kill switches such as this are helping to drive these next generation cell therapies to the clinic by improving safety to the patients.

P0776

Augmenting expansion and tumor infiltration of chimeric antigen receptor T cells with a novel dendritic cell vaccine for solid tumors

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Chimeric antigen receptor T (CAR-T) cell therapy faces challenges in treating solid tumors, particularly due to poor tumor infiltration and inadequate expansion. Here, we present T-booster, a novel dendritic cell (DC) vaccine co-engineered with tumor antigen receptor (TAA) and immunostimulatory cytokine and chemokine, which significantly augments the efficacy of CAR-T cell therapy against solid tumors. Firstly, Claudin18.2 (CLDN18.2) modified T-Booster and CLDN18.2 targeting CAR-T were used for proof of concept. In vitro assays demonstrated T-booster's ability to significantly increase CAR-T cell activation, proliferation, and cytotoxicity. In vivo studies utilizing both conventional and large-refractory gastric cancer xenograft models demonstrated that the combination of T-booster with CAR-T cell therapy resulted in complete tumor regression and prolonged survival, without incurring additional toxicity. Mechanistic studies elucidated that the modification of DC cells leading to their preferentially homing to the tumor site, thereby establishing a microenvironment enriched with these cytokines and chemokines, which are responsible for chemoattracting CAR-T. Additionally, CAR-T cells stimulated with T-booster exhibited reduced exhaustion markers and increased markers for T cell activation, memory formation, and apoptosis resistance. Furthermore, T-booster expressing EpCAM was shown to enhance the in vitro and in vivo efficacy of EpCAM-targeting CAR-T cells, underscoring

its potential as a platform technology for various TAA. Collectively, our findings suggest that T-booster may represent a novel strategy to enhance anti-tumor efficacy of CAR-T cell therapy in solid tumors.

P0777

A programmable and automated microfluidic platform for massively parallel and sequential processing of single cell assay operations

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Recent advancements in cancer immunotherapy have revolutionised the treatment paradigm and significantly improved clinical outcomes for patients. Single cell profiling has been pivotal in the development of these immunotherapies. However, while traditional single cell technologies have yielded extensive 'omic datasets enabling users to infer biological function, they have not yet empowered the direct functional analysis at single cell level. We propose that such analysis is crucial for understanding the complex cellular interactions within the tissue microenvironment. To address this challenge, we introduce a novel platform that leverages droplet microfluidics and optical electrowetting-on-dielectric (oEWOD). This platform enables highly-controlled, sequential and multiplexed single cell assays in massively parallelised workflows. This capability enables functional profiling of single cells in a scalable screening format. Soluble reagents, cells, or assay beads are encapsulated into droplets in fluorous oil. These droplets are actively filtered based on size and content, ensuring only desirable droplets (e.g., single cell droplets) are retained for analysis, thereby overcoming the challenge of Poisson distribution, a limiting factor with many other single cell technologies. The droplets are arrayed on a temperature-controlled chip, and each droplet's history is tracked from filtering to workflow completion. On the chip, droplets undergo an automated suite of operations, including merging and multi-colour fluorescent imaging. This enables precise execution of complex sequential assay workflows and multiparametric assay readouts providing functional insights that are not possible with many other single-cell approaches. To illustrate the platform's utility in immunotherapy research and development, we present single-cell functional workflows for antibody discovery and cell and gene therapy. We describe generation of droplets containing single immune effector cells, such as T-cells, and single target cells, which can be merged sequentially and repeatedly to test effector cell killing efficacy, specificity and exhaustion. In summary, our droplet-based approach empowers direct functional analysis at the single-cell level, advancing our understanding of cellular interactions and cellular function and accelerating progress in cancer immunotherapy research and development.

P0778

Advancing CAR-NK Cell Immunotherapy: Scalable Production of Natural Killer Cells from Induced Pluripotent Stem Cells Using Bioreactors

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The expansion of Natural Killer (NK) cells from peripheral blood (PB) or umbilical cord blood (UCB) faces limitations, notably donor-to-donor variability, which impacts cell yield and cytotoxic profiles. To address these challenges, we are setting up a scalable NK production platform leveraging induced pluripotent stem cells (iPSCs) as the starting material. This approach may mitigate donor dependency and ensure reproducibility in NK cell production.

We evaluated the feeder-free expansion of NK cells isolated from Buffy Coat samples obtained from different donors. Initially, NK cells isolated via negative selection were pre-expanded in static G-Rex culture plates and subsequently transferred to the Xuri Wave (W25) bioreactor. However, we observed highly variable results in terms of fold-expansion during the G-Rex pre-expansion phase, specifically an average of 6.8 folds, with a standard deviation of 7.07. This variability limited the subsequent expansion phase in the Xuri bioreactor, highlighting the necessity for a more standardized approach. On the other hand, we successfully cultured human induced pluripotent stem cells (iPSCs) derived from both fibroblasts and CD34+ cells. We tested two different differentiation protocols for iPSCs to generate NK cells. Quality controls included flow cytometry panels designed to monitor pluripotency and differentiation progress over time. In vitro, iPSCs consistently maintained more than 95% of positivity for SOX2, Oct3/4, TRA-1-81, NANOG, SEEA4, and CD30. However, these pluripotency markers were lost upon differentiation, while cells acquired a CD34-positive, and subsequently CD56-positive phenotype.

Overall, we have successfully expanded NK cells in a bioreactor and set the protocol for deriving them from iPSCs. Currently, our focus is on integrating these two processes and combining them with a clinical-grade gene editing approach.

P0779

Enhancing the yield of allogeneic, “off-the-shelf” TCR-negative CAR T cell production via in-process purification using CD3-CAR NK-92 feeder cells

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Chimeric antigen receptor (CAR) T cells have shown great potential in treating hematological malignancies. However, the production of patient-derived CAR T cells is both time-consuming and costly, leading to a growing interest in allogeneic CAR T cells as an “off-the-shelf” alternative. Despite their potential, the production is limited by manufacturing complexity, scalability issues, and the risk of Graft-versus-Host disease (GvHD) due to remaining allo-reactive T cell receptors

(TCRs)/CD3+ cells in the CAR-T product. To address these challenges, we developed a scalable manufacturing method for TCR/CD3-negative CAR T cells using non-viral CRISPR-Cas9 knock-in and a purifying CAR-equipped NK-92 feeder cell line.

The T-cell receptor α constant (*TRAC*) locus is a common target for non-viral knock-in of a CAR and simultaneous knock-out of the endogenous TCR. However, even a low percentage of remaining TCR/CD3+ cells can lead to GvHD, with clinical trials reporting GvHD after a single infusion of 0.1-1.1% TCR+ cells. Our approach involves two consecutive cocultures with gamma-irradiated CD3-specific CAR NK-92 cells for an in-process purification. They effectively eliminate residual TCR/CD3+ T cells to less than 0.01%, representing a 45-fold improvement compared to the commonly used magnetic cell separation (MACS) method. The irradiated, short-lived CAR-NK-92 cells not only increase the purity of the CAR T product but also have a feeder effect, which leads to a 5-fold increase in CAR T cell yield. Additionally, the inherent MACS-associated cell loss as well as the costs for MACS reagents can be overcome. We estimate that by applying our CAR-NK-92 purification strategy, manufacturing cost can be lowered by 65%. The resulting *TRAC*-replaced CAR T cell products maintain their cytotoxic activity and a favorable T cell phenotype. We now also demonstrate that the same approach can be applied to *CD3-zeta* knock-in CAR T cells, emphasizing the flexibility of the platform to expand purified allogeneic TCR-negative CAR T cells generated by different methodologies.

With the recent advances in the CAR T cell field and the emphases of developing allogeneic therapeutics for millions of newly diagnosed cancer patients annually, our method represents a significant advancement for in the field of scalable, “off-the-shelf” CAR T cell therapy.

P0780

Gene Editing induced Gene Silencing (GEiGS®) technology to develop TME-responsive macrophages for solid tumour immunotherapy

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In solid tumours, the presence of tumour associated macrophages (TAM) is generally associated with poor prognosis. TAMs are known to supress response to standard-of-care therapeutics, including chemotherapy, immunotherapy (CAR-T) and angiogenic inhibitor. An immunosuppressive tumour microenvironment (TME) is the primary reason that limits the efficacy of cell therapy approaches. The extent to which macrophage supports or inhibits tumour growth is linked to their response to cues from the TME.

Here we present a novel TME-responsive macrophage cell therapy approach for treatment of solid tumours, based on cells engineered with a new gene silencing technology called Gene Editing induced Gene Silencing (GEiGS). GEiGS recodes endogenous miRNAs to redirect them towards new targets, and in this application takes advantage of phenotype-specific miRNA expression patterns to control macrophage phenotype in response to TME cues.

GEiGS-engineered macrophages use TME cues to turn on a pro-inflammatory programme once they reach solid tumour site. We present data on how GEiGS can be used to control the (pro- and anti-inflammatory) function of iPSC-derived macrophages in a programable manner using phenotype-specific miRNA expression pattern to selectively silence the target gene. Furthermore,

we utilise state of the art in-vitro models to demonstrate how gene silencing can be linked to extracellular cues within the TME, facilitating development of cell therapies with both improved efficacy and safety profiles. The technology is broadly applicable to autologous and both donor-derived and iPSC-derived allogeneic approaches.

This establishes GEiGS as a powerful new approach for gene silencing which enables the generation of programable therapeutic products, and opens new avenues in cellular engineering in the immuno-oncology space. We believe this validates a core platform to develop next generation, adaptive cell therapies, addressing many challenges with current approaches.

P0781

Advances in CAR T cell microarray screening for off-target identification

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The development of cell therapies has been a crucial progression of cancer treatment, with six CAR-T cell therapies approved by the FDA to date alongside movement into other indications such as autoimmune diseases. However, CAR-T cell therapy developers must continue to address side effects and limitations, such as cytokine release syndrome, high relapse rate, and efficacy against solid tumours. Here we describe recent developments in cell therapeutic screening capabilities using Charles River's Retrogenix® Cell Microarray Platform, to support the development of safe, targeted, and viable cell therapies.

The Retrogenix® Platform Library contains over 6,500 full length human plasma membrane, secreted, and heterodimeric proteins that are over-expressed in Human Embryonic Kidney-293 (HEK293) cells and screened for interactions with test articles. This technology can be used for a wide range of complex therapeutics to identify primary receptors and de-risk potential off-target interactions against the human and non-human cell-surface proteome expressed in their natural cellular environment, resulting in highly specific/relevant data with rapid turnaround, and low false positive and negative rates.

Using the Retrogenix® Platform to elucidate CAR-dependent interactions can help to identify potential risks associated with undesirable off-target binding and allow mitigation by downstream assessments such as functional assays. Recent developments in the screening technique for cell therapies includes the reduction of cell numbers required for screening, while maintaining a high degree of sensitivity. This aids the feasibility of using the Retrogenix® Technology to de-risk cell therapies that are expensive to produce and difficult to expand.

Here, we demonstrate use of this platform to identify CAR-specific interactions and non-specific T-cell interactors, using CAR T cells generated as part of the integrated, end-to-end capabilities for cell therapy discovery, safety, and manufacturing at Charles River. Additional work providing functional assessment of these cells is also highlighted. These capabilities and platform developments allow thorough assessment of the binding and subsequent function of therapeutic candidates, aiming to support favorable outcomes at later-stage safety testing and into the clinic.

P0782

Fast-track system-level analysis of human therapeutic T cell and CAR-T signaling pathways and in vivo functions

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1: JC Discovery

Chimeric antigen receptor (CAR) T cell therapy has emerged as a revolutionary treatment for hematological malignancies, but its adaptation to solid tumors is impeded by the inhospitableness of the solid tumor microenvironment (TME). Recent advances in genetic editing allowed multiplex gene manipulation of T cells, promising the development of turbocharged CAR T cells with improved durability, tumor-killing capabilities, as well as resistance to the hostile TME. Despite the profound successes in hematological malignancies, solid tumors present intricate challenges that necessitate innovative solutions. Along that line, we will present a constellation of approaches aiming at deciphering through interactomics the mode of action (1) of drug and biologics aiming at enhancing CAR T cells and (2) of detrimental coinhibitory receptors or negative intracytoplasmic regulators. Moreover, our ability to simultaneously analyze via omics the CAR-T cells, the myeloid and lymphoid cells and the tumor cells from several anatomical sites – including the TME - in a single mouse allows an unprecedented analysis of the efficacy of CAR-T cells in vivo following infusion or direct in vivo engineering, Therefore, the fast-track system-level analysis of human T cell and CAR-T signaling pathways described here should be favored to optimize CAR T cell signaling and evaluate, at the systems level, the mechanisms of action and possible side effects of drugs or genetic modification aiming at improving CAR-T and therapeutic T cells before translating them into the clinic.

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P0783

Transduction and metabolic-driven primary T-cell expansion in novel scalable bioreactors

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The expansion of cell populations ex-vivo is key in cell-based therapies, from process development to the manufacturing of therapeutic doses. For optimal growth, fresh media supplementation is often required. Conventional media exchange is performed at set feeding intervals based on reproducible practice. However, this strategy assumes that cells, even those of the exact same type and source, grow under precisely the same conditions at any given time. In reality, cells are influenced by multiple factors such as the environment and operator variability.

Metabolic-driven feeding regimes have shown value in maintaining optimal culture conditions. From a data analytics standpoint, coupling metabolite data during culture expansion provides a feedback loop that generates a more accurate understanding of cell culture in real time. Metabolite data, such as glucose and lactate levels, provide important clues about cell culture, allowing the user to adapt and customise media exchange strategies.

Coupling data analytics with more frequent media exchanges requires custom cell culture vessels specifically adapted for this purpose. We leverage novel microfluidics-influenced bioreactors and compare different feeding strategies, such as media addition, continuous perfusion, and media replacement, using Jurkat cells (T-cell line) and primary T-cells. Optimal conditions resulted in 2-5x cell expansion (depending on the media exchange strategy) in these custom bioreactors compared to conventional systems.

These bioreactors are also scalable while maintaining their form factor. Keeping the bioreactor geometry consistent across scales ensures that fluidic streamlines affecting the cell microenvironment remain similar, resulting in identical cell conditions in both scale-down and scale-up approaches. Moreover, these bioreactors permit high-efficiency cell transduction by taking advantage of the high surface area-to-volume ratio (S/V). Following transduction and an in-situ wash step, the same system is used to expand cell populations. This results in larger numbers of high-quality transduced cells, enhancing overall yield and avoiding the need for different vessels and lab instruments.

There is immense potential for these novel bioreactors to leverage automation for process development, running multiple parallel experiments, thus providing labor and reagent cost savings. Coupled with analytics, the metabolite feedback output can be integrated with automation to consistently record data and adapt protocols to the different cell expansion stages.

P0784

Regenerative medicine and cell therapy: maturing you knowledge with Livecyte

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1: Phasefocus - Bruker

In vitro studies are key starting points for developing effective cellular therapies, both for regenerative medicine and immunotherapies. Whether observing engineered immune cells or progenitor and iPSCs, the ability to investigate cell behaviour without causing harm to cells is of utmost importance from early-stage discovery to clinic.

Livecyte's Quantitative Phase Imaging is a powerful label-free live-cell imaging technique, generating high-contrast, fluorescence-like images without the risk of phototoxicity or cytotoxicity associated with fluorescence labelling. The enhanced contrast increases the

robustness of single-cell segmentation and tracking whilst maintaining true physiological conditions over a long period of time.

Through Livecyte you can assess the health of your iPS cells and colonies and the health of cultures through colony morphology, growth rate and number of differentiated single cells. As these cells differentiate Livecyte can identify population heterogeneity evaluating cellular characteristics ranging from morphology, motility, to proliferation and growth to gain in depth insights into cell differentiation. Livecyte has also set a new precedence for neuronal differentiation with neurite outgrowth assays. Neuronal cells are more at risk of phototoxicity reducing imaging over long periods of time and limiting the ability to look at network formation dynamically. With Livecyte's advanced analysis recipe, neurite outgrowth can be quantified from the initial changes in cell morphology and migration through to cells forming attachments and network formation. Metrics such as the number of branching points and total neurite length can be obtained, gaining a novel understanding of the effect of therapeutic drugs on the dynamics of neurite outgrowth. More recently Livecyte has been used in a clinical environment to monitor spinal cells prior to patient implant.

In addition to stem cell therapy, T-cell based immunotherapy is an exciting emerging tool in the fight against cancer, with *in vitro* T-cell killing assays being the first step in validating the effectiveness of new therapies. However current methods only tell a small part of the story, revealing only population-based metrics of cell death at particular timepoints, or at best looking at these death events kinetically. With no scope for cell-cell interaction kinetics or monitoring target cell proliferation and growth, this leaves a large gap in knowledge on why certain treatments are clinically more efficacious than others. In an attempt to yield more information, current assays commonly depend upon time intensive manual tracking, or use of fluorescence imaging of T-cells, which are often primary cells, and highly sensitive to phototoxicity as a result. However, with unlabelled T-Cells, Livecyte can gain a plethora of T cell interaction kinetics with target cells including the time and number of T cell contacts with a target cell before cell death occurred without having to label T cells giving more meaningful insights on how your engineered T cells are behaving and more predictive information for in vivo studies.

P0785

Accelerating innovation in chimeric antigen receptors: Harnessing mRNA for rapid screening

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Chimeric antigen receptor (CAR) T cell therapies represent a revolutionary advance in oncology, but are currently approved for only a limited number of cancers. This highlights the urgent clinical need for the development of novel CAR T cell therapeutics. While the diverse and customizable modular nature of CARs allows for specific tailoring to different tumor types and immune cell specifications, it also complicates the identification of an optimal lead construct for later clinical use.

Traditionally, CAR constructs are tested by viral transduction of immune cells, followed by assessment of the killing efficiency of the resulting CAR immune cells on target cells. This study presents a novel screening system that facilitates the rapid and cost-effective generation and evaluation of multiple CAR constructs. Its innovation and novelty lies in the use of mRNA and non-viral gene transfer, in conjunction with a smart construct design, which allows normalization

of CAR expression. The system allows multiple parameters, such as transfection efficiency, CAR expression, and CAR signaling, to be determined in parallel using a specifically generated reporter cell line. This facilitates multiplexing, automation, and high-throughput analysis of multiple CAR constructs.

This innovative platform promises to accelerate the development and optimization of CAR cell therapies, paving the way for their wider application in clinical oncology.

P0786

CARTAR: a web tool for identifying and prioritizing CAR therapy candidate targets using TCGA and GTEx data

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Chimeric Antigen Receptor (CAR) therapy has transformed cancer treatment by employing engineered immune cells to specifically target and destroy cancer cells. This is an expanding field in which plenty of researchers are working, however it is limited by the identification of suitable targets. Herein, we introduce our in-home developed CARTAR webserver offering a comprehensive suite of tools for the specificity and selectivity analysis of potential CAR targets. To showcase the utility of CARTAR, we applied the tool to identify potential CAR strategies for treating pancreatic adenocarcinoma (PAAD), a prevalent and lethal cancer.

Using expression data from The Cancer Genome Atlas (TCGA) and the Genotype-Tissue Expression Project (GTEx), CARTAR is developed to identify tumour-associated antigens, ensuring precise target selection, evaluating specificity to avoid off-tumour toxicities, and aiding in the rational design of dual CARs. Furthermore, the webserver enables users to explore candidate target expression in cancer cell lines utilizing data from the Cancer Cell Line Encyclopedia (CCLE), facilitating in the identification of cell lines suitable for testing the activation and cytotoxicity of designed CAR cells. As a result of this analysis, one conventional (targeting CEACAM6) and one logic-gated CAR (targeting S100P and KRT17) strategies are proposed for the treatment of PAAD.

To the best of our knowledge, CARTAR stands out as the first webserver dedicated to the systematic search for suitable candidate targets in CAR therapy. It holds immense potential as a valuable resource in the clinical immunotherapy landscape and its capability to predict success of FDA approved CAR targets has shown its potential in the field. CARTAR is freely accessible at https://gmxenomica.github.io/CARTAR/.

P0787

UniCAR T-cell potency – the higher the affinity of the UniCAR to the adaptor molecule, the higher the potency?

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In recent decades, various strategies have been implemented to reprogram immune effectors against tumour cells. Among them, Chimeric Antigen Receptor (CAR) T-cell therapy has shown remarkable efficacy in haematological malignancies. However, this therapy can also cause severe to life-threatening side effects. To address these safety concerns, we have developed modular CAR platforms, such as the Universal CAR (UniCAR) system. In the UniCAR system, T-cells are genetically modified to express a single-chain fragment variable (scFv) targeting the E5B9 UniCAR epitope derived from the nuclear La protein. UniCAR T-cell redirection to the target cells relies on a Target Module (TM), a bridging molecule that contains the E5B9 epitope and a tumour-specific binding moiety. Appropriate UniCAR-T activation thus involves two interactions with different affinities: between the TM and CAR T-cells, and the TM and target cells. To date, little is known about the role of the affinity between adaptor CARs and adaptor molecules on CAR T-cell functionality. In the UniCAR platform, the interaction between the UniCAR and the TM can easily be fine-tuned by modifying the primary amino acid sequence and size of the original E5B9 UniCAR epitope, without the need to re-engineer the CAR. In this work, we investigate whether and how alterations of the amino acid sequence of the E5B9 UniCAR epitope, including the amino acids flanking the E5B9 epitope in the native La protein, may impact the affinity of the UniCAR to the TM and thereby the functionality of the adaptor CAR system. In this way, we identified potential new UniCAR related epitopes and among them, one named E5B9L, for which the monoclonal antibody 5B9 has the highest affinity. Having replaced the original E5B9 UniCAR epitope with E5B9L in a TM targeting the Fibroblast Activation Protein (FAP), we evaluated how this modification affects UniCAR T-cell functionality. The binding properties of the newly generated anti-FAP-E5B9L TMs to UniCAR and their capacity to redirect UniCAR T-cells were compared directly with those of the original anti-FAP-E5B9 TMs. In spite of the significant variation in the affinity of the different TMs to the UniCAR, no major differences were detected with respect to cytotoxic and cytokine-release properties of the redirected UniCAR T-cells. Altogether, our findings show no significant impact on the *in vitro* functionality and potency of UniCAR T-cells, indicating that increasing the affinity between the UniCAR and the TM by changing the amino acid sequence of the E5B9 UniCAR epitope does not play an essential role in this adaptor CAR system.

P0788

Optimizing exosome production from CAR-T cells for cancer immunotherapy

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Chimeric antigen receptor (CAR) T cell therapy has revolutionized immunotherapy, offering a promising approach for treating various conditions. However, significant challenges remain, including cytokine release syndrome and neuroinflammation, as well as complications like tumor lysis syndrome and graft-versus-host disease. Moreover, current CAR-T cells are not efficient for the treatment of solid tumors due to antigenic diversity, immune escape within the tumor microenvironment, and the challenge of achieving sufficient proliferation and infiltration.

Exosomes derived from CAR-T cells (EXO-CAR-T) can offer a complementary therapy due to the unique properties of extracellular vesicles (EVs). These EVs, including exosomes, are capable of mediating intercellular communication and carrying a repertoire of therapeutic molecules. By using EXO-CAR-T, we hypothesize that we can mitigate some of the limitations and side effects of traditional CAR-T cell therapy. Exosome-based therapies offer biocompatibility, low toxicity, high permeability, and stability in biological fluids, with the ability to cross biological barriers and be engineered for targeted drug delivery. However, the large-scale preparation of exosomes from CAR-T cells is expensive, and the safety and immunogenicity of engineered exosomes need careful consideration.

Our research focuses on several key areas:

1. Exosome Biogenesis and Production: Through gene editing techniques, we aimed to improve the production yield of exosomes from CAR-T cells. Our preliminary data showed an increase in exosome output.

2. Safety and Immunogenicity: To address concerns regarding the safety and immunogenicity of engineered exosomes, we analyzed the effects of eliminating HLA-I expression to produce allogeneic EXO-CAR-T.

Our preliminary findings suggest that exosomes derived from CAR-T cells represent a promising complementary therapy for cancer treatment, particularly for solid tumors. By enhancing exosome production and engineering them to evade immune detection, we can potentially overcome many of the limitations associated with traditional CAR-T cell therapy.

P0789

HCPure process related impurity clearance from viral vectors

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Final product purity is essential to the efficacy and safety of cutting-edge cell and gene therapies. Removal of host cell proteins (HCP) from the therapeutic target is therefore an essential step in cell and gene therapy development. To achieve the purities required, intermediate and polishing steps are often employed in multistep purification processes. For such steps, mixed-mode chromatography can be a powerful tool, utilising both ionic exchange and hydrophobic interaction binding interactions to maximise purification potential and therefore reduce processing costs. We present a novel mixed-mode chromatography resin, designed for the removal of host cell proteins from a range of targets and expression systems. The application of this mixed-mode resin for polishing purification strategies is demonstrated, with purification performance illustrated for different therapeutic targets and expression systems under a range of loading conditions.

P0790

Continuously improving quality and efficiency in AAV manufacturing

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1: IDT Biologika

This work explores continuous improvements of quality and efficiency in AAV manufacturing processes. Operating within non-GMP and research-focused environments, the study places a significant emphasis on consistently optimizing both the quality and efficiency of AAV processes, which can be transferred easily into a GMP setting. Utilizing IDT Biologikas' proprietary HEK293 cell line, which we benchmarked against commercially available AAV manufacturing solutions, we are investigating how our platform approach is responding to ongoing advancements in AAV manufacturing.

While implementing a generic manufacturing platform for AAV´s we evaluate a broad set of upcoming technologies and trends that might potentially redefine quality attributes, safety profiles and contribute to reducing serious adverse effect. In this Talk we will report about IDT´s journey in implementing a generic concept for AAV manufacturing and about the current standards in production processes, giving a future perspective on the upcoming activities, while evaluating new available technologies from a GMP manufacturer point of view.

The exploration of new technologies in AAV manufacturing strives to set new benchmarks. A ready-to-use platform, along with cutting-edge techniques and a proprietary cell line, become instrumental in achieving sustained quality and efficiency. In particular we will allude on further optimization in AAV manufacturing by integrating the use of small molecules and plasmids technologies and the implementation of post-translational modifications characterization by use

of analytical solutions beyond the current regulatory requirements. Thus potentially supporting the definition of standards for quality and efficiency for gene therapeutic products.

P0791

USP Technology platform to accelerate gene therapy bioprocess development

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1: Sensorion

Gene therapies, based on the use of AAV, take more and more place in the range of the therapies proposed by the Biotech pharma. There are various AAV production systems (adherent vs suspension cells; baculovirus/sf9 cell system, transient transfection, stable cell line ...) used depending of the genetic disease treatment and clinical/commercial needs. The bioprocess development based on transient transfection in HEK 293 cells from setting parameters at small scale to process optimization at large scale has to be repeated for each new therapeutic candidate and is often outsourced by lack of in-house equipment, manpower and state of the art. Moreover, if the biotech does not have the internal CMC capabilities, the biotech is dependent on the service provider in terms of slot availability. To have an in-house bioprocess development laboratory is a real advantage to overcome the drawbacks of the outsourcing. At Sensorion, we built an USP/DSP bioprocess development laboratories. The USP fully-equipped facilities include a dedicated cell culture room for cell thawing/freezing and cell amplification and some specific equipment such as bioreactors of different sizes, a cell counter and a metabolite analyser. This platform which allows to perform early step of bioprocess development by means of DOE, define the optimal process parameters/conditions and scale up the AAV production process up to 50L scale in single use bioreactor will be presented. Furthermore, thanks to 2L and 10L Twin reusable glass bioreactors, the platform enables to produce and supply very quickly small batches to supply internal analytical development and preclinical studies. With our platform, we are able to develop USP and DSP processes and achieve the tech transfer and scale up to our industrial partner for manufacturing clinical batches.

P0792

GET-IN: The Gene Therapy Innovation Training Network

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Gene therapy has transitioned from a distant hope to reality. To date, five gene therapies based on rAAV have been approved in the EU, with over thirty phase III clinical trials currently ongoing, and promising advancements in therapeutic gene editing on the horizon. However, barriers and fundamental limitations in the manufacturing process of gene therapy vectors and delivery

systems limit broader application. While the current bioprocessing technology is adequate to support small-scale production for localized gene therapies, the high cost of materials and resources, and the lack of scalability, robustness and an open process environment limit commercial viability. In addition, the process is not sufficiently efficient and requires several log-fold scaling, to support producing high doses for whole body treatment and/or to treat large-patient groups. In line, novel and improved vector approaches to increase specificity, efficacy and functionality at lower doses are needed to tackle the emerging safety concerns and high costs observed in current clinical trials. Additionally, improved humanized models to reduce attrition rates through the development pipeline are urgently needed.

Innovation in gene therapy requires multidisciplinary approaches. GET-IN is a doctoral network of 7 academic and 8 non-academic partners coordinated by KU Leuven and supported by the European Union's Framework Programme for Research and Innovation. The network unites expertise in vectorology, genome editing, process engineering, biological manufacturing and innovative humanized disease models. Together, they provide an excellent training framework for 10 Doctoral Candidates as future innovators in the gene therapy field. Research in GET-IN addresses significant knowledge gaps that currently hinder the widespread adoption of gene therapies. This involves optimization of scalable up-stream and down-stream processes, which includes enhancing the production through bioengineering and developing industry-standard biomanufacturing practices. Additionally, GET-IN aims to discover novel, safe, and efficacious gene editing tools broadly applicable, which will be combined with innovative delivery methods. Finally, we will develop cutting-edge organ-on-chip technology that allows for the evaluation of efficacy, target tissue specificity, and toxicity in humanized models, surpassing current animal model standards.

The joint training program emphasizes responsible, cooperative research and innovation, creativity, and entrepreneurship to maximize the career potential of the Doctoral Candidates.

P0793

End-to-end manufacturing of autologous CAR T cell therapies with the new Sefia™ cell therapy manufacturing platform

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1: Cytiva

Gene-modified T cell therapies have shown clinical efficacy in targeting cancer, with promising results in treating other diseases. However, the commercialization of these breakthrough therapies remains challenging due to the increased need for research and clinical studies. To facilitate scalable, flexible, and cost-effective solutions, we have developed the Sefia™ cell therapy manufacturing platform. This modular, automated, and functionally closed technology consists of the Sefia Select™ system for T cell magnetic isolation, expanded cell harvest, and final formulation and the Sefia expansion system, designed to activate, transduce, and expand chimeric antigen receptor (CAR) T cells.

Frozen (n = 3) and fresh (n = 3) leukopaks from healthy donors were purchased from Charles River and CytoCare, respectively. They were processed using the Sefia Select™ system for magnetic

CD4/CD8 isolation. Isolated T cells (1×10^8) were seeded on the Sefia expansion system, activated with T Cell TransAct reagent on day 0, transduced with CAR-LVV on day 1, and expanded using a recently developed serum-free T cell medium (Akron). Cell recovery, purity, viability, and phenotype were quantitated on days 0, 5, and at harvest. Further processing was performed on the Sefia Select™ system and VIA Freeze™ instrument for final product formulation and freezing. Post-thaw, CAR T cell functionality was evaluated via an *in vitro* killing assay.

The Sefia™ cell therapy manufacturing platform can expand > 2×10^9 T cells by day 7–9, with a total fold expansion of 30.18 ± 7.6, a viability of 97.5 ± 0.01%, and a CAR-LVV transduction efficiency of 55.3 ± 0.1%. With an average cell recovery > 85%, the final product represents more than the maximum therapeutic dose needed for a patient.

The Sefia™ cell therapy manufacturing platform provides an end-to-end solution for autologous CAR T cell therapy manufacturing. This modular and digitally integrated platform combines two closed systems with dedicated application software and single-use kits to automate manufacturing from starting samples (fresh or frozen apheresis) to final patient doses.

P0794

Large-scale Expansion of Human Mesenchymal Stem Cells in Xeno-free and Serum-free Conditions

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1: FUJIFILM Irvine Scientific

The development of biotherapeutics requires a robust and reproducible manufacturing process. When using human mesenchymal stem cells (hMSCs), the process needs to minimize cell stress and handling, while maximizing differentiation potential and cell growth in scalable systems.

FUJIFILM Irvine Scientific is launching the industry's first off-the-shelf MSC basal media and frozen supplement in a single-use closed system format, lowering unit operation costs and increasing scalability for commercial applications. Using a rational design approach, the xeno- and serum-free medium (XSFM) was developed. This medium performs well in static culture systems and has been adapted for use in a 5 L bag for large-scale closed culture systems. PRIME-XV MSC XSFM Dual Component kit is a combination of MSC basal and supplement, and the bag format keeps the critical, temperature-sensitive supplement frozen until ready to use. The 5 L bag of MSC basal media is packaged and shipped at 2-8 °C. Pellethane tubing on both bags allows for easy connection by aseptic welding and mixing. The tubing is compatible with the Terumo BCT welders, including the TSCD-Q sterile tube welder; however, the additional PVC line included facilitates compatibility with other welders.

The PRIME-XV MSC XSFM Dual Component kit is fully compatible with the Quantum bioreactor. It supports a yield of over $2x10^8$ total MSCs from an initial seed of $2.5x10^7$ cells in seven days of expansion and helps maintain the stemness of human adipose-derived MSCs post-expansion. The dual-component bag format enhances product stability and reduces the need for freezer storage while maintaining sterility for closed-system applications in MSC manufacturing and MSC-based therapy.

P0795

A Novel Technology for Accurate Quantification of Viral Genome and Determination of Lipid Nanoparticle Encapsulation Efficiency

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1: Promega

This research evaluates the efficacy of Adeno-associated viral (AAV) vectors and Lipid Nanoparticles (LNP) as delivery systems, focusing on overcoming existing challenges in genome titering for AAV and encapsulation efficiency for LNP. Both delivery modalities have shown significant potential, yet they encounter specific technical challenges that impact their efficacy and reproducibility. We introduce a novel approach using TruTiter Reagent, a membrane impermeable molecule that binds to nucleic acids (DNA or RNA) and covalently conjugates with them, enhancing the accuracy of genome titering and encapsulation measurements.

AAV vectors typically require DNase treatment to remove extraneous DNA, followed by proteinase K treatment to release viral genomes resistant to DNase. However, DNase treatment often results in incomplete digestion and does not access DNA in partially ruptured viral capsids. We developed a new method using TruTiter Reagent, which excludes interference by AAV particles at concentrations below 100µM. This method involves treating the sample with 10µM TruTiter for 30 minutes, allowing it to crosslink free nucleic acids or those from damaged viruses, followed by neutralization and proteinase K treatment. The effectiveness of this approach was tested by comparing traditional DNase methods with the TruTiter method using AAV serotypes (AAV2, AAV9, AAV8) treated under various conditions. Results demonstrated that TruTiter offers a more robust and consistent quantification of genome titers, evidenced by reduced standard deviation in measurements compared to DNase.

For LNPs, encapsulation efficiency is crucial for determining optimal lipid composition and understanding storage conditions. The TruTiter Reagent effectively isolates and protects the nucleic acid content within LNPs, thereby facilitating more accurate efficiency assessments. We investigated this by subjecting GFP mRNA encapsulated in LNPs to different temperatures, noting that TruTiter indicated significant reductions in encapsulation efficiency with increased temperatures, whereas PicoGreen showed minimal change. Further experiments with in vitro transcribed RNA and various transfection reagents confirmed the superiority of TruTiter in detecting actual encapsulation efficiency, crucial for effective LNP design and application.

This study highlights the importance of developing more precise analytical techniques for improving the reliability of viral and non-viral delivery systems. The adoption of TruTiter Reagent represents a significant advance in addressing the variability and technical challenges previously encountered with traditional methods, paving the way for enhanced development and deployment of gene therapy and vaccine delivery platforms.

P0796

Inducible expression systems for AAV production in mammalian cells

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For the production of recombinant adeno-associated virus (AAV) in mammalian cells, a triple transfection method using three different plasmids is commonly used. Two plasmids provide the AAV genes *rep*, *cap* and the adenoviral (AdV) helper genes. The third plasmid contains the sequence encoding the therapeutic gene to be packaged into the viral particles. Packaging cell lines, that stably express all AAV and AdV components have the advantage that only one plasmid encoding the target gene must be transfected, which markedly increases production efficiency. However, the expression of cytotoxic proteins such as the AAV gene *rep* in stable cell lines requires a tight regulation of gene expression. Therefore, an inducible expression system is being developed that comprises viral polymerases from bacterial as well as mammalian viruses. These are the T7 bacteriophage, the +ssRNA virus Semliki Forest virus (SFV) and the -ssRNA virus vesicular stomatitis virus (VSV). The inducible systems are based on the specific interaction of a viral RNA polymerase and the corresponding viral promoter. Thus, expression is activated only when a plasmid encoding the viral polymerase is present in the cell. Therefore, we propose an antibiotic-free alternative to the widely used TetON system.

In our study, we investigate the feasibility of new inducible systems in HEK293. Therefore, we designed and introduced different plasmids carrying fluorescent proteins under the control of the viral promoters T7, VSV and SFV. As a benchmark, the TetON system was included and evaluated in our study. Basal and induced expression levels after co-expression of the corresponding RNA polymerase were assessed by flow cytometry.

Subsequently, the best performing viral inducible system was compared to TetON in stable cell lines using the recombinase-mediated cassette exchange (RMCE) for reproducible genome integration. When stably integrating genes for AAV production under control of a viral promoter there were no or only minimal expression levels detected. Growth rates and behaviour were comparable to HEK293 without integrated genes. Non-integrated genes for AAV production were provided by plasmid transfection. Our experiments demonstrate the activation of protein expression using viral polymerases and contribute to the development of stable cell lines for the expression of toxic proteins in mammalian cells.

P0797

Assessing genetic plasticity in HEK293 – Comparative analysis of HEK293 cells in response to selective pressures

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Human embryonic kidney cells (HEK293) serve as cell factories in viral vector manufacturing, particularly for recombinant adeno-associated virus (rAAV) production. Despite their widespread utilization for industrial applications, a comprehensive characterization of the HEK293 genome and epigenome stability remains elusive, mirroring concerns observed in other immortalized cell lines such as Chinese Hamster ovary (CHO) cells. To address this knowledge gap, this study employs a systematic approach to examine the genetic and epigenetic landscapes of HEK293 cell lines with the objective of evaluating their responses to changing environmental conditions and improving the current understanding of how these molecular mechanisms might influence rAAV production processes. Therefore, adherent HEK293 cells were adapted to suspension growth using various commercially available serum-free media formulations. Following successful adaptation, whole-genome and methylome deep sequencing was performed on both adapted and parental cell lines. The sequenced reads were then aligned to the human reference genome, enabling the assessment of genome stability, structural variants, and DNA methylation patterns. Comparative analysis of the different cell lines revealed that changes occurred at all observed levels over the course of adaptation. Alterations in the distribution of structural variants, including single nucleotide polymorphisms or insertions and deletions could be observed as a response to the adaptation process to suspension growth conditions, indicating a dynamic nature of HEK293 cells at the genetic level. Overall, this work offers novel insights into the cellular responses of HEK293 cells to selective pressures and lays the groundwork for more comprehensive omics characterization, which could be employed to develop cell lines achieving higher production efficiency.

P0798

Unlocking the power of transient transfection: Advancing rAAV production with standardized protocols and complex characterization

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1: Roche

AAV vector production in human suspension cell culture using transient transfection is a state of the art technology for the production of viral vector based gene therapies. In these processes, primarily HEK293-derived cell lines are transfected with plasmids containing the genetic information for viral vector production. The key challenges of these production processes are characterized by poor process robustness and scalability arising from the high complexity and variability of the transfection unit operation. This process inefficiency results in high production costs, therefore restricting availability of life-saving rAAV-based gene therapies for patients in need.

To address these challenges, we evaluated critical transfection parameters, such as complex size and complexation time, utilizing methods such as dynamic light scattering (DLS). This approach allowed us to generate data illustrating the relationship between transfection complex size and final titre, thus enabling the establishment of an optimized transfection protocol that is scalable from benchtop up to 500-liter scale. It is our firm belief that this in-depth characterization of the transfection complex formation will accelerate the path towards more robust and scalable rAAV production processes, ultimately serving the needs of patients.

P0800

Validation of an automated method for nucleated cell count and viability determination

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In the context of the quality control of Advanced Therapy Medicinal Products (ATMPs), the evaluation of cell count and viability is highly critical both because it is involved in various decision-making phases of the cell manufacturing and because it affects the production yield as well as the final dose preparation.

Therefore, the method used for the measurement of cell count and viability needs to be strongly reliable for the analysis of nucleated cells since it is applied both to manufacturing process control and to the final drug product characterization for release.

Different methodologies can be applied, among them manual counting by haemocytometer or automated technics such as flow cytometry and digital imaging of stained cells.

The digital imaging of stained cells (DISC) represents the preferred choice in our cell factory since it allows easy and accurate measurements in a timely manner, in compliance with the peculiar needs of GMP quality controls for ATMPs.

DISC is based on a closed, fully automated safe and no toxic cell counting system that allows the automation of dye-exclusion methods overcoming the human bias that would influence the analysis result. The image obtained is digitalized and the instrument software provides the number of dead or live cells. In our context, DISC is compatible with 21 CFR Part 11/GMP guidelines.

A DISC method to evaluate cell count and viability of nucleated cells was validated for dilution linearity, reproducibility and accuracy in relation to Burker haemocytometer and flow cytometry.

The validation results showed that DISC method is highly reproducible and accurate when compared with reference methods such as manual counting and flow cytometry.

P0801

Enhancing rAAV manufacturing through Raman spectroscopy-based process analytical technology

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In recent years, gene therapy has witnessed significant strides with the regulatory approval of various products. Among these, recombinant adeno-associated virus (rAAV) has emerged as a promising vector for targeted gene delivery. However, realizing the full potential of rAAV in clinical applications necessitates the development of a robust manufacturing process capable of consistently producing high-titer, high-quality products. Despite notable progress in rAAV manufacturing tools, further enhancements are essential to meet growing demands and ensure

widespread accessibility of gene therapy products. Process Analytical Technology (PAT) offers a promising avenue for advancing the characterization and efficiency of rAAV manufacturing. By harnessing advanced analytical techniques like Raman spectroscopy, we can achieve real-time monitoring and control of critical process parameters. Raman spectroscopy is a powerful spectroscopic method that measures inelastic light scattering across different frequencies, providing a unique molecular fingerprint based on analyte composition and concentrations. In this study, we employed in-line Raman spectroscopy to collect spectral data from multiple rAAV lab-scale production batches. Leveraging Partial Least Squares (PLS) multivariate analysis, we developed empirical models to detect and quantify key bioreactor growth parameters, including glucose, lactate, and viable cell density (VCD). Through careful calibration and validation, our models demonstrated high accuracy and robustness in estimating metabolite concentrations and cell density in real-time. These results underscore the potential of Raman spectroscopy coupled with multivariate analysis as a valuable tool for precise monitoring and control of the rAAV manufacturing process. By facilitating timely interventions and optimization, this approach holds promise for enhancing product quality and meeting the evolving demands of gene therapy applications.

P0802

Controlling empty and partially filled capsid impurities using zonal ultracentrifugation in cGMP large scale rAAV production

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A robust, reliable and scalable purification process for recombinant Adeno-associated virus (rAAV) vectors is essential to the gene therapy industry and to date, manufacturing purification methods for rAAV vectors are mostly based on affinity chromatography, for (rAAV) particles capture, and ion-exchange for full particle enrichment.

These purified vector preparations may still require a final polishing step to remove impurities in the form of vector subtypes. The downstream purified capsid preparations will generally consist of populations of; fully packaged vectors, partially packaged and empty vectors. In addition, resolution of over packaged vectors should be accommodated in a process.

Purified rAAV vectors occur due to the packaged genome sizes, including full-genome, intermediate, and empty particles. As empty capsids are thought to reduce transduction efficiency and can induce unexpected immune responses, there is an exponentially increasing demand for improved purification strategy with the aim of lowering the empty particles content to properly support rAAV-based preclinical and clinical studies. In addition, IEX ion exchange chromatography steps can be time-consuming to develop because they depend on the serotype being used.

Using preparative ultracentrifugation which has the capacity to be useful in cGMP manufacturing of gene therapies at large scale we investigated the resolving power of a range of centrifugation techniques namely, continuous flow gradient separation, batch mode gradient separation and high resolution gradient separations. The aim was to resolve the particle sub-types in a single density gradient operation, removing the need for two or more rounds of centrifugation which is time and resource consuming.

Ultracentrifugation relies on the density difference between the filled particles and the contaminating particle sub-types which come about due to; over filling, partial filling or non-filling of particles in the production process. In this study affinity chromatography purified product is subjected to ultracentrifugation using a range of techniques to further resolve the purified rAAV into populations defined as; overfilled, filled, partially filled and empty capsids.

The three techniques offer versatility for production scale; continuous flow loading of large volume samples resulting in filled capsids separated from partially filled capsids, batch loading of smaller samples with resolution of filled capsids from partially filled and overfilled capsids and a high resolution high capacity technique to separate filled from overfilled and partially filled capsids where partially filled capsids can represent a significant proportion of the load.

P0803

Separation of full and empty particles of adeno-associated virus using an improved method and condition on anion exchange chromatography

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1: Osaka University 2: U-Medico

Adeno-associated virus (AAV) vectors are important tools for gene therapy, but the vector manufacturing remains challenges. One of the challenges is the large formation of particles devoid of the transgene (empty particle) compared with the desired therapeutic product (full particle). The concerns about empty particles on clinical risk and AAV-mediated transgene expression have required the development of a robust and scalable purification process to separate empty particles. Anion exchange chromatography (AEX) is a useful method for separating empty and full particles at large scale because of its scalability. Here, we showed the improvement of chromatography method and buffer condition in the AEX process to enrich full particles. It is likely that our chromatography method and condition provides several advantages over the traditional method.

P0804

Optimizing viral vector technology transfer: Data and science driven fast track and modular framework approach

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1: Charles River Laboratories

At Charles River Laboratories, we have harnessed decades of experience in viral vector contract development and manufacturing to create a robust, adaptable technology transfer frameworks essential for safeguarding the development of gene therapy programs. Viral vectors manufacturing, pivotal in gene therapies, demand unparalleled expertise and meticulous execution to prevent costly delays and ensure regulatory compliance. Our comprehensive

technology transfer program is designed to support developers at any stage of their journey, ensuring seamless technology transfer to Charles River's viral vector center of excellence in Maryland, US.

In this poster, we will present how we have developed the technology transfer frameworks and how this can support the cell and gene therapy ecosystem. We will detail these frameworks as integral parts of our methodology and will demonstrate how comprehensive, data and science driven technology transfer approach facilitates efficient, reliable, and scalable manufacturing solutions, safeguarding the continuity and success of gene therapy projects in an evolving regulatory landscape.

Based on the analyses of 50+ technology transfers performed at Charles River, we have developed two technology transfer frameworks: Fast Track and Modular. The Fast Track framework facilitates rapid transfer and pathway to GMP of well-established viral vector processes, including stages such as knowledge transfer, assay transfer, fast track manufacturing, quality control testing, and regulatory support. This approach is suitable for maintaining critical production timelines when no process modifications are required.

The Modular framework offers a flexible, customizable approach for processes requiring further development or adjustments. It allows developers to select specific modules tailored to their unique project requirements, such as cell line development, plasmid supply, process development/optimization runs, comparability/bridging studies, and analytical development. This modularity provides the right level of flexibility to facilitate a technology transfer while de-risking a pathway to GMP.

Standardized protocols and templated documentation ensure consistency and accuracy in data collection and process transfer. Specialized teams of process and analytical development, and MSAT (Manufacturing, Science & Technologies) professionals work closely with therapeutics developers to align project goals and mitigate risks through each transfer stage. This comprehensive approach maximizes the probability of success and reduces the risk of potential delays both in the clinical and commercial stages of the drug development.

Allowing Charles River Laboratories to present this poster at the European Society for Cell and Gene Therapy Congress will provide attendees with valuable insights into our innovative and reliable approach to viral vector technology transfer, showcasing the practical implementation and success of our methodologies in supporting the cell and gene therapy ecosystem.

P0805

Evaluation of a novel HEK cell line for transient transfection for viral vector manufacturing

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1: AGC Biologics

AGC biologics is a leading global CDMO, providing world-class development and manufacture of mammalian and microbial-based therapeutic proteins, plasmid DNA, viral vectors and genetically engineered cells.

Our expertise includes the production of Adeno-Associated Vectors (AAV) through transient transfection in HEK293 cells, with both adherent and suspension cultures, and applicable in ex-vivo and in-vivo gene therapies. Moreover we have a long lasting experience in Lentiviral Vector (LVV) manufacturing through transient transfection in HEK293T cells, with both adherent and suspension cultures. AGC Biologics has developed the BravoAAV™ platform, enabling the production of AAV at various scales and adaptable to different serotypes and ProntoLVV™ platform for the manufacturing of LVV.

To further elevate our competitive edge and service portfolio, AGC Biologics has entered into a strategic partnership with Cytiva to test the ELEVECTA™ HEK293-derived transient cell line. This collaboration focuses on assessing the productivity of this cell line when subjected to transient transfection with a reporter transgene. Notably, this cell line is the precursor of ELEVECTA™ producer cell line, designed to incorporate all necessary genes for AAV production, and is easily inducible to produce AAV. The transient transfection approach delivers rapid results at small scales and during early development phases, offering valuable insights into AAV productivity before transitioning to a stable, inducible producer cell line. Moreover the same line has been tested for LVV manufacturing.

Productivity results and full/empty capsid ratio obtained transfecting the ELEVECTA™ HEK293-derived transient cell line compared to the standard HEK293-derived transient cell line will be shown.

These promising results have prompted AGC Biologics to evaluate both packaging and producer ELEVECTA™ cell lines. This initiative aims to offer clients enhanced capabilities for AAV and LVV production, leveraging Cytiva's cutting-edge platform.

P0806

Affinia’s proprietary AAV plasmid design produces industry leading manufacturability of novel and WT capsids

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Identification of novel AAV capsids with improved tropism will unlock many additional indications for gene therapy. While the increased tropism may result in lower doses, there is still a need to have increased yields and packaging efficiency to ensure a robust, scalable manufacturing process with a lower cost per dose. We have found that performing a manufacturing assessment for yield, process fit and stability during the lead identification stage in screening leads to more commercially viable capsids arising from the screen. Here we will show data from these manufacturability screens for our myotropic and BBB-penetrant capsids. This data gives us confidence that our lead capsids will perform well as they progress into clinical development. Additionally, we have developed a robust process development toolbox that can further improve yields, packaging efficiency and stability.

Our proprietary plasmid design increases our yields and packaging efficiency (% full capsids at harvest) for all novel capsids with peptide inserts and WT serotypes tested to date. Interestingly, we have seen that this plasmid design improves yield, packaging efficiency and residual DNA of

novel capsids better than AAV9. For novel capsids we can achieve yields greater than 3e12 and packaging efficiency over 60% while for AAV9 we can achieve yields greater than 4e12 but packaging efficiency of only greater than 40%. Our proprietary plasmid designs lead to industry leading yields and packaging efficiency for our novel, peptide insert capsids.

P0807

Achieve higher AAV productivity with the next-generation transfection reagent *Trans*IT-AAViator™

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In the rapidly advancing field of cell and gene therapy, manufacturing recombinant AAV therapeutics has historically been inefficient. Older legacy processes often yielded only a handful of doses for a medium-high dose patient treatment, underscoring the need for more efficient manufacturing methods. Today, AAV therapies necessitate both high titers and high-quality viral genomes per dose, making the optimization of AAV production processes critical to reducing overall costs and improving accessibility. This challenge limits scalability and accessibility, driving up the costs of these therapies. Mirus Bio is excited to announce the launch of *Trans*IT-AAViator, a next-generation transfection reagent to meet these challenges. *Trans*IT-AAViator features an optimized lipid and polymer formulation that requires less plasmid DNA and transfection reagent (by volume), leading to improved AAV productivity and a better workflow. This synthetic and animal-origin-free transfection reagent was designed to be used alongside *Rev*IT™ AAV Enhancer to fully maximize the ability to increase AAV titers and percent full AAV capsids in different AAV serotypes. The result is a robust, scalable system that can yield fold improvements in titer and percent full AAV capsids to boost the efficiency of AAV upstream workflows. These improvements lead to fold increases in the number of therapeutic doses produced per bioreactor and therefore significantly lower the cost per dose. The *Trans*IT-AAViator AAV production system thus represents an advancement in the field, offering a solution to the most persistent challenges in AAV therapeutic development.

P0808

Lentiviral vector-based polymeric nanoparticles as gene and cell therapy product - A cross-functional approach to streamline and control a complex manufacturing process

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Intracellular delivery of nucleic acids to mammalian cells using non-viral gene delivery remains a challenge both *in vitro* and *in vivo*, with transfections often suffering from variable efficacy and limited stability. Moreover, physicochemical properties of nano-sized materials play a significant role on the *in vivo* fate of nanomaterials. Particle size, size distribution, global charge or shape are defined as the key parameters by health agencies for the manufacturing quality as well as the safety and efficacity of nano-sized particles.

Alaya.bio's platform is a unique technology that combines the versatility of cationic and shielding polymers as biodegradable and safe transfection material together with the gene transfer efficiency of lentiviral vectors (LVs). Here we report on the cross-functional approach we have implemented to control and optimize manufacturing process parameters of a complex nanoparticle-based therapeutic product.

Scalable and GMP-compatible bioprocesses have been engineered for the production of LVs lacking the VSV-G immunogenic protein. Process performance and repeatability have been demonstrated by verifying the integrity and functionality of viral particles (DLS, interferometry, sandwich ELISA, RT-qPCR, flow cytometry and qPCR). A portfolio of 40 families of cationic and shielding biodegradable and pH-responsive polymers (with diversified shape, MW and pKa) has been assembled and screened to formulate homogeneously sized LV-based nanoparticles (Dh<300 nm and PdI<0.3) that efficiently deliver *in vitro* transgenes to different types of human cells that are relevant for the development of gene and cell therapies.

Since many factors influence the coating of LVs by polymers such as active and inactive ingredient concentration, buffer composition and pH, mixing and incubation time, we took advantage of high throughput DLS and *in vitro* transduction assays to optimize the formulation development. Coating of the best functional nanoparticles has been scaled-up and tested for compatibility with microfluidics-based formulation in order to secure the translation towards industrial manufacturing. This iterative process based on the same orthogonal methods to characterize the LV raw material down to the final nanoparticle was instrumental in the refinement of the space design around our product.

By combining optimized bioproduction of lentiviral vector payload and high throughput screening of formulations with superior biophysical and functional properties, Alaya.bio has now access to next generation nanoparticles that will be applied to rapid and universal manufacturing of CAR T-cells with preserved naive and memory phenotypes.

P0809

How minicircle DNA helps to overcome current shortcomings in the field of gene and cell therapy – an overview

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In the past few years, there have been notable advancements in gene and cell therapy that show immense potential for transforming the healthcare industry. Progress in new ATMPs (advanced therapy medicinal products) e.g., with innovative gene editing technologies provide promising novel approaches to treat cancer, genetic disorders or autoimmune diseases and raise hopes for numerous patients.

However, secure and approachable cell and gene therapies all rely on safe and reliable gene delivery. Unfavourably, conventional delivery systems can come along with a variety of hurdles e.g., a high DNA toxicity (regarding generation of CAR-T cells using plasmid DNA), retro-packaging events (regarding AAV production) or certain safety risks due to biased genome integration (regarding modifications of T-cells with lenti viral vectors). These issues cause high regulatory effort, high costs and restrict the efficiency of ATMP development and production, altogether limiting the worldwide availability of cell and gene therapies.

The Minicircle has emerged as a Next Generation Gene Vector consisting almost exclusively of the sequence of interest and associated elements required for the application in the target cells (<150 bp), therefore, offering great potential for maximizing efficiency while minimizing risks. The lack of bacterial sequences and the compact, small structure showed to be beneficial for various applications. It was successfully used for diverse approaches from cell engineering, viral vector production to RNAi. This poster aims to provide an overview of the characteristics and applications of PlasmidFactory's minicircle DNA and how it can help to unlock the full potential of gene and cell therapy and pave the way for personalized medicine.

P0810

Get the full control of your T cell isolation for your CAR-T therapy manufacturing

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Cell therapies offer endless potential treatments for currently untreatable malignancies. Approaches such as chimeric antigen receptor (CAR)T cell therapies are being evaluated in numerous clinical trials with a number of them emerging as approved therapies, particularly in hematological tumors. Due to the growing numbers of these therapies and the complex workflow required to produce them, additional tools are needed to produce a consistent product in a closed, automated process. The Sefia Select™ system is a functionally closed solution that has been developed to enable automation of key processing steps within the CAR-T cell workflow. MagnetSelect application software is an expanded offering for the Sefia Select system to enable full automation of magnetic T cell isolation as part of the first step of cell therapy manufacturing. This MagnetSelect application provides flexibility to adapt the automated process to specific needs as well as consistent and high biological performances independent of the initial cell population variability.

P0811

Viral Sensitizers unleash host cell potential to enhance AAV production

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Elevating production yield to meet the demands of patients is imperative for viral vector-based gene therapy products. This study focuses on overcoming cellular self-defense mechanisms, such as antiviral cellular processes, which impede reaching the cell's full potential.

We utilized Virica Biotech's Viral Sensitizers (VSEs), small molecules that transiently alter cell-signalling pathways, favourably influencing the production of the Adeno-associated viral (AAV) vector. We tested different VSE compounds in certain concentrations on HEK293 cells to assess their toxicity range and effectiveness in producing AAV2. The scale-down method was implemented using 96-deep well plates and verified in 125-ml shake flasks. BalanCD HEK293 served as the basal growth medium, and the addition of VSEs occurred one hour before transfection. Consequently, the addition of screened VSEs in properly tailored concentrations improved AAV2 titer up to three to four folds compared to the control, while maintaining cell viability within the range of the control.

Improving the yield of viral vectors remains a critical challenge within gene therapy. This study highlights the effectiveness of integrating small molecule compounds known as Viral Sensitizers (VSEs) into the existing production process utilizing chemical transfection to substantially enhance AAV viral vector production in the HEK293 cell line.

P0812

Impact of chromatographic support, load sample preparation and elution conditions on full AAV particles enrichment

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1: Sensorion

Over the last years, gene therapy involving recombinant adeno-associated virus (AAV) vectors holds great promise for treating a wide range of genetic disorders, such as congenital hearing loss. The manufacturing of a safe and high quality therapeutic product remains a major challenge, despite the emergence of technical innovation among which affinity resin dedicated to the capture of a large panel of AAV serotypes. One main product-related impurity consists of particles devoid of genetic material, and decreasing the proportion of these so-called empty capsids, requires fine tuning of the downstream process development. The implementation of such polishing step within an AAV manufacturing process relies on the balance benefit-risk. Indeed each AAV therapeutic candidate (serotype/transgene) has a different chemical behavior needing identification of specific conditions to reach satisfying results. A case study using different ion-exchange chromatographic supports, POROS™ 50 HQ resin formed of small particles packed into a column (Thermo Fisher Scientific) and CIMmultus® QA monolith formed of hollow cylinders whose surface walls hold functional group (Sartorius), to target full AAV particles enrichment will be presented. The impact of the load sample preparation (dilution vs buffer exchange) and the elution conditions (salt screening, gradient vs isocratic) on the separation of empty and full capsids as well as on the step recovery will be discussed. Based on small-scale development on the POROS™ 50 HQ resin, the combination of magnesium chloride and magnesium sulfate in the elution buffer as well as an isocratic elution lead to resolutive peaks and a satisfying separation allowing for a 4-fold enrichment in full particles. The CIMmultus QA monolith combined with sodium acetate elution leads to higher vector genome recovery and around 2.5-fold enrichment in full AAV particles.

P0813

MACS® GMP Nuclease A: A new clinical-grade endonuclease specifically developed for highly reliable production of retroviral vectors in the manufacturing of cell and gene therapy products

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The generation of lentiviral vectors used in the context of cell product manufacturing and field of gene therapy poses strict requirements with regard to the purity of the vector. Lentiviral vectors are released into the supernatant of transfected HEK293T cells. This process is prone to host cell nucleic acid as well as plasmid DNA contaminations. Typically, non-specific endonucleases are used to remove these impurities during the virus purification process, however, these enzymes themselves bear the potential to additionally transmit host cell protein (HCP), DNA or endotoxins to the vector.

Furthermore, endonucleases are widely applied in the beginning of downstream processing (DSP) procedures of biologics to reduce viscosity, enabling easier handling and more efficient protein extraction through the removal of DNA and RNA.

We have therefore developed a clinical-grade endonuclease A, the MACS® GMP Nuclease A, combining all required features necessary to be applied for the manufacturing of especially cell therapy products. This is accomplished by the combination of the following attributes:

- Manufactured and tested under quality management system (ISO 13485), in compliance with relevant GMP guidelines
- Animal- and human material free manufacturing
- Highest purity
- Low endotoxin contamination
- High and defined activity optimized for virus purification processes
- Low *E.coli* HCP content
- Tested for sterility according to Ph. Eur. (Chapter 5.2.12)
- Long-term stability at various temperatures
- LC/MS-based identity confirmation

This product fits perfectly into various production upscale formats, ranging from 1-200 L, by offering three different product sizes of 100 K, 1 M and 4 M Units.

We show the benefits of MACS® GMP Nuclease A application in viral vector production resulting in high level lentiviral vector titers with high purity, indicated by low residual total and plasmid DNA, endotoxins and residual endonuclease. The functionality study confirms the highest quality manufacturing in comparison to a state-of-the-art product.

P0814

Implementing Virus Filtration to Ensure Pathogen Safety of Cell and Gene Therapy Products

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Ensuring pathogen safety is critical in the manufacture of biological products to reliably provide safe medicines to patients. Production of cell and gene therapy (CGT) products provides new challenges for the assurance of pathogen safety compared to traditional recombinant products due to raw materials which can pose higher risk of virus contamination and downstream purification steps which provide limited capability to remove or inactivate potential virus contaminants. One of the most effective and robust unit operations for removal of viral contaminants is virus filtration. Unfortunately, the very size-based mechanism which leads to its high degree of virus removal also makes virus filtration difficult to implement for many CGT manufacturing processes. Here, we performed virus spiking studies to evaluate removal of a model virus contaminant from a parvovirus-containing feed utilizing a 35 nm pore size filter. The data show robust virus removal and excellent product yields even when challenged with various filtration fluxes and with interruptions in the process. Additionally, case studies will be shown demonstrating the applicability of virus filtration in AAV manufacturing process. Finally, we will discuss strategies and feasibility for the incorporation of filtration as an upstream barrier for protection of other gene therapy and cell therapy products as well. Overall, the increased risks associated with new therapeutic modalities call for implementation of the robust protection capability that only virus filtration can provide.

P0815

miniVec: improving safety and performance for ATMP developers using next-generation vectors

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1: VectorBuilder

VectorBuilder's proprietary miniVec™ plasmid offers a miniaturized backbone, providing remarkable efficacy, safety, and manufacturability for cell and gene therapies. Comparing to traditional plasmids, miniVec™ plasmids have higher plasmid manufacturing and virus packaging yields, improved transgene expression, and an enhanced safety profile.

The miniVec™ backbone supports antibiotic-free and supplement-free selection. miniVec™ plasmids can be applied across diverse expression systems, including lentivirus, AAV, in vitro transcription (IVT), non-viral regular plasmids, piggyBac, and Sleeping Beauty.

miniVec™ is an ideal choice for piggyBac and Sleeping Beauty transposon systems due to its streamlined design, reduced metabolic burden on host cells, and enhanced safety profile. These features allow miniVec™ to achieve comparable or increased transposition efficiency in cells.

miniVec™ plasmids can achieve higher manufacturing yields compared to traditional plasmids due to several key mechanisms including a high-copy-number favored fermentation, more efficient replication, and a reduced metabolic burden.

P0816

Effective expansion of NK, TIL and gamma delta T cells utilizing genetically modificed irradiated feeder cells

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1: Jiangsu Hillgene Biopharma Co., Ltd

Introduction

Natural killer (NK) cells, tumor infiltrating lymphocytes (TILs) and gamma delta T cells have showed the great therapeutic potential for treat solid tumors and chronic diseases. Due to their low immunogenicity, these cell types are expected to be developed into universal cell products to treat patients at low dose and re-dosing regime. However, large-scale cell expansion in a bottleneck for manufacturing allogeneic NK and gamma delta T cell therapies and autologous TIL thearpies. Studies have shown that genetically modified K562 feeder cells can promote the activation and proliferation of some ex vivo hard-to-grow cell types including NK, gamma delta T and TILs. As feeder cells, irradiated K562 cells can only secrete soluble growth factors and stimuli to activate and proliferate target cell growth and gradually die in culture, and can be washed away in the final cell theraoy product. Therefore, biosafety of using K562 feeder cells is not a major concern. Many NK cell and iPSC-NK cell therapies produced using the feeder-based processes have been approved for clinical trials worldwide. China regulatory has approved some cell therapies produced uisng feeder cells processes for clinical trials, mainly in the fields of TIL cells, NK cells and iPSC-NK cell therapies. Therefore, the development and production of genetically modified K562 feeder cell has a great commerical value.

Key technologies and methods

The genetically modified K562 cell line development is complex and consists of number of steps including (1) target gene modified molecule design; (2) gene modified K562 cell polyclonal construction; (3) gene modified K562 cell monoclonal screening; (4) gene modified K562 monoclonal cell library construction and library identification; (5) cell library stability study; (6) GMP K562 feeder cell banking and characteristics; (7) gene modified K562 feeder cell process development and production; (8) genetically modified K562 cells will then be GMP manufactured at large scale and irradiation before QC for product release; (9) product stability study and (10) in vivo carcinogenicity testing.

Conclusion

The genetically modified irradiated K562 feeder cells have been well-developed and manufactured in Hillgene. The RUO- and GMP-grade K562 feeder cell products are now available on the market to support the whole cycle of cell therapy product development from discovery, pre-clinical, clinical and commercial stages.

P0817

Vial Containment Systems for Gene Therapies

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Gene therapies based on viral vectors typically are stored at ultra-low temperature (ca. -80°C, or container with dry ice at ca. -78°C). There are three requirements of the vial containment system (vial, elastomer stopper, seal): (a) maintain container closure integrity (CCI) performance through fill/finish operations, storage/distribution, and thaw, (b) present no adverse interaction with gene therapy, and (c) provide adequate recovery and functionality of vector, whether adeno-associated virus, adenovirus, or lentivirus. Understanding performance against these requirements is essential for a manufacturer to decide if a vial containment system is suitable.

The present research was a long-term study of the performance of vial containment systems comprising cyclic olefin polymer (COP) vials, bromobutyl elastomer stoppers [with ethene tetrafluoroethene (ETFE) laminate film on surface that faces drug product] and aluminum flip off seals. Comparators were vial containment systems comprising borosilicate glass and polypropylene.

CCI performance evaluation (laser-based headspace analysis) was made of vial containment systems stored at -80°C for up to two years. No ingress of oxygen was observed over one year and only a small amount (5%) was observed over 2 years (likely through permeation of COP vial). There was no system breach. In the case of storage on dry ice for 7 days, after 30 minutes at room temperature no ingress of CO_2 was observed; after 3 days CO_2 ingress at 2.5% was observed. Ingress can be reduced substantially with a poly(ethylene terephthalate)-based secondary container.

Potential interaction with gene therapy was evaluated in two ways. The first was by exposure of model proteins to COP vials, borosilicate glass vials, and various stoppers under agitated conditions and measuring the levels of particles formed and protein loss – both indicators of unwanted interaction that may be extrapolated to virus capsids. The COP-based system provided for substantially reduced interaction. The second was by comparing (headspace gas chromatography) stoppers with and without ETFE film for preventing migration of components from elastomer. Presence of ETFE film substantially reduced migration. Systems comprising COP vials and stoppers with ETFE film provided enhanced performance.

Virus recovery and functionality were evaluated after storage at -80°C and thaw for selected adeno-associated viruses, adenoviruses, and lentiviruses. The system comprising COP vials and stoppers with ETFE film performed better than a borosilicate-based system, and equivalent, within error limits, to a standard polypropylene screwcap vial system.

Anticipating growth of gene therapies, COP vials are now offered in a nested vial in tub format that facilitates use in an automated filling line.

All factors considered (CCI performance, reducing risk of potential interaction, virus viability), vial containment systems comprising COP vials and stoppers with ETFE laminates may offer advantages for the storage of virus-based gene therapies versus systems comprising glass or polypropylene.

P0818

Cyclic Olefin polymer based vial systems for cell and gene therapy

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As cell and gene therapy (CGT) drug products are becoming more mainstream, primary packaging consideration for these classes of therapies are evolving and increasingly gaining regulatory attention. When selecting a primary packaging solution, it is imperative to understand the critical quality attributes of an ideal containment system, including low particulate levels, container closure integrity (CCI), ice nucleation, drug-material compatibility, and ease of fill-finish.

Particulates come in many forms. Unlike traditional biologics, the active ingredients of CGT products (e.g., viral vectors, cells) are considered particles themselves. On the other hand, particulates other than the intended active ingredient, which can be of visible and subvisible sizes, or be of intrinsic (e.g., cellular debris) or extrinsic origin (e.g., environmental contaminants, raw materials, consumables), can accumulate in the drug product from manufacturing through cold chain all the way to the point of care. Using light microscopy and flow imaging microscopy, cyclic olefin polymer (COP) vials were found to have similar or fewer subvisible particles, compared to ISO standard glass vials, polypropylene (PP) screw cap vials and ethylene vinyl acetate (EVA) bags, with or without freeze-thaw, upon ultra-cold (-80°C) or cryogenic (≤ -150°C) storage.

Product sterility depends on the primary container being able to maintain CCI. Containers with proper CCI prevent the ingress of low-temperature gasses, over pressurization of the container, and consequent hazard to its handlers. O_2 headspace analysis and He leak test revealed that COP vials can maintain CCI upon proper crimping after short-term (1 week) or long-term (24 month) cryogenic storage.

Ice nucleation is a critical process parameter for the cryopreservation of biological materials composed of sensitive membrane structure. Controlling ice nucleation within a tolerable range of temperatures and time is important to avoid loss of the high-value material and its potency. Our study presented a new method of measuring ice nucleation that captured a greater extent of critical process deviations in a be packaged aqueous system than the conventional method.

Drug-material compatibility is an important aspect to protect the drug product integrity as any interaction could result in its degradation resulting in reduced potency and/or generation of harmful by-products. Here we show the compatibility of COP vials with different cell types which upon cryo-storage for short-term showed optimal functionality in different tested assays.

Additionally, COP based CZ vials are packaged in a nested tub configuration and designed to meet selected exterior dimensions for ISO glass standard **8362-1, are s**terile, ready-to-use offer broad compatibility with automated and flexible fill finish systems for CGT drug products as well as other advanced therapies.

We hereby emphasize the importance of primary packaging for CGT manufacturing and demonstrate the advantage of COP vials as a primary packaging system in the various technical considerations. The compatibility of CZ vials with different cell types along with its availability in different sizes and packaging formats offers a convenient and efficient containment solution for small batch fill/finish operations for CGT applications.

P0819

Maintaining superior viral vector recovery in cell and gene therapy applications by using Daikyo Crystal Zenith® vials

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1: West Pharmaceutical Services

This study aims to evaluate the impact of vial material of construction on the stability of two adeno-associated virus (AAV) serotypes under varying stress and storage conditions. AAV8 and AAV9 were chosen as representative AAV serotypes, with respective titers in a common buffer formulation, and evaluated in different 2mL vial types: Daikyo Crystal Zenith® (CZ) vial, and two commercially available borosilicate vials. To simulate viral vector recovery in cell and gene therapy applications, the vial combinations were tested across five challenge conditions:

1. 3 months at -80C, recommended condition for long term storage

2. 2 months at 5C, recommended condition for temporary refrigerated storage

3. 3 weeks at 25C, simulating room temp clinical ambient conditions

4. 72 hours of 250RPM agitation, to mimic handling during manufacturing/shipping

5. 5 cycles of Freeze/Thaw, to mimic handling during manufacturing and the potential for structural damage to proteins or DNA

In all conditions, the Daikyo CZ vial demonstrated improved viral vector recovery compared to borosilicate glass vials. The data shows that there were no significant changes in product quality due to container storage in the Daikyo CZ vials, and that the CZ vials may comparatively improve viral vector recovery across common storage and handling conditions.

P0820

Development of alkaline-tolerant affinity chromatography resin with immobilized AAV receptor protein and optimization of anion-exchange chromatography conditions for AAV full particle enrichment

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Adeno-associated viruses (AAVs) are promising vectors for in vivo gene therapy. Cellular entry of various AAV serotypes is mediated by an interaction with the AAV receptor, AAVR (KIAA0319L). Utilizing this protein, we have developed an affinity chromatography resin to which modified AAVR is anchored.

Since wild-type AAVR is not stable for an affinity ligand, we have been improving its stability using directed evolution. Our AAVR mutant exhibited high stability in acidic conditions, which allows AAVR-AAV binding in neutral pH and subsequent elution of AAV vectors in acidic conditions. This acid-tolerance enables the repetitive use of this affinity resin without loss of AAV binding capacity.

In this presentation, we report a development of alkaline-tolerant AAVR for preparative affinity resin. Stability in alkaline condition is desired for alkaline cleaning-in-place (CIP), which is conducted to wash out strongly bound host-cell proteins after use of column. Using the acid-

tolerant AAVR as a staring sequence, we have successfully developed an alkaline-tolerant AAVR mutant. The affinity resin with immobilized alkaline-tolerant AAVR maintained chromatographic performance without significant loss of AAV binding capacity after 100 sets of alkaline CIP. This alkaline tolerance not only mitigate the risk of contamination by alkaline CIP, but also lead to a reduction of the overall cost of AAV purification by allowing the reuse of affinity resin. Furthermore, the affinity column packed with this alkaline-tolerant AAVR resin accomplished purification of AAV8 vector from clarified harvest with a recovery yield of more than 90%. The purity of eluted AAV samples was found to be on a par with other commercially available affinity columns.

In addition to the AAVR affinity column, we report anion-exchange (AEX) chromatography for the enrichment of full AAV vector that contains a transgene in its capsid. While a significant amount of empty AAV vectors, devoid of a transgene, can be produced in the AAV manufacturing, empty vectors cannot be removed in the affinity chromatography due to their identical capsid surface structure. AEX chromatography can be used to remove AAV empty vectors by utilizing the difference in electrostatic charge between full and empty particles. We utilized choline chloride as an eluting salt, which exhibits lower biological toxicity than other quaternary ammonium salts such as tetramethylammonium chloride. The optimized separation condition using AEX resin TOYOPEARL GigaCap Q-650S accomplished enrichment of full AAV8 particle from the mixture of full/empty particles (input: 35%, output: 88%).

In summary, we have developed affinity chromatography resin onto which AAVR mutant is immobilized. This affinity resin exhibited high durability in both acidic and basic conditions, thereby maintaining high AAV binding capacity after repetitive use. Additionally, optimized AEX condition allowed removal of empty capsids from the mixture of full and empty particles. We believe that our AAVR affinity column and TOYOPEARL GigaCap Q-650S are conducive to the development of downstream process for AAV manufacturing.

P0821

Development of a scalable downstream process with AEX polishing for AAV8 vectors delivering 50% vg recovery and 3-fold enrichment of full AAV capsids

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Facing the increasing demand for AAV vectors supporting pre-clinical and clinical programs, the manufacturing process for AAV8 vectors was scaled up to 50L pilot production. The platform is based on suspension cell line transiently transfected with three plasmids system, using clinically relevant transgenes, relying on scalable technologies with single-use bioreactor and empty/full AAV capsid separation based on anion-exchange (AEX) chromatography.

Process development on critical unit operation such as AAV affinity capture at small-scale led to more than 2-fold increase in vector genome (vg) recovery. Two critical process parameters (CPP) were identified for AAV8 capsid: loading density and elution conditions with the CaptureSelect™ AAVX affinity technology. The process changes applied showed a significant improvement in vg recovery from 40% up to 90% demonstrated on 0.2ml micro-columns. The optimal process parameters were applied along up-scaling returning comparable vector recovery above 90% at 50L pilot scale for two independent AAV8 vector batches.

Process development tackled another critical unit operation, establishing scalable and reproducible empty/full AAV capsid separation. The process characterization was performed through (i) bottom-up purification study using internal reference material purified by CsCl density gradient and (ii) top-down purification study using design-of-experiment (DoE) approach with scale-down 1ml CIM QA column loaded by mixture empty/full AAV8 post-affinity. The results shown conflicting outcomes between recovery and purity responses. The factors combination of minimizing vector loading density onto the stationary phase while operating toward alkaline pH region led to optimal responses with vg recovery of 50% and full AAV capsid enrichment factor close to or above 3-fold (from 35% to 100% vg/cp ratio measured by qPCR/ELISA). Lastly, AEX polishing was further scaled-up to pilot operation using 400ml CIM QA column for two independent AAV8 vector batches. Purity after AEX was ranging from 87% to 104% measured by vg/cp ratio and vg recovery measured between 46% and 55% by qPCR targeting ITR.
To demonstrate process reproducibility, two additional batches delivered vg recovery up to 62% within the statistical model limits. The overall drug product quality was further investigated using orthogonal methods, including mass photometry showing % full capsid up to 49% for the recent campaigns. Other impurities residuals analysis showed HEK293 host cell protein (HCP) below 20 ng/ml in the pilot scale AAV8-based drug products.

Overall, the accelerated process development project led to establishing and up-scaling an end-to-end AAV8 manufacturing process with compressed timeline while demonstrating reproducibility, consistent quality, predictable yield and competitive productivity.

P0822

High-throughput plasmid DNA isoform analysis using capillary gel electrophoresis

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Plasmid DNA represents an integral starting material in cell and gene therapy applications. Manufacturing of plasmids is commonly performed using *E.coli* during upstream processing, followed by alkaline lysis, chromatographic purification and ultra/diafiltration in downstream processing. Plasmids exist in different isoforms, namely the covalently-closed-circular (ccc), open-circular (oc) and linear (lin) isoform, with the latter two being considered product related impurities. The manufacturing process is steered towards a high purity of the ccc isoform, relying on high quality analytical data and rapid turnaround times. Capillary gel electrophoresis coupled with laser-induced fluorescence detection (CGE-LIF) can fulfill these requirements. While commercially available kits employ coated capillaries and proprietary separation gels (M Wang et al., 2021), we developed a method using uncoated capillaries and commercially available polymers based on literature (LA Holland et al., 2022). The ccc, oc and lin isoforms, as well as oligomers of the ccc isoform are baseline separated within 10 min. The method has been successfully used for plasmid sizes ranging from 4 kbp to 12 kbp. The ccc-isoform purity exhibits a ≤ 1% relative standard deviation. A limit of quantification of 1.0% is routinely achieved. Scalability of the method to a multi-capillary system for parallel sample analysis can be explored.

P0823

Rapid and inexpensive purification of adenovirus vectors using optimised aqueous two-phase technology

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Adenovirus vectors are used in several laboratories to treat human diseases and have also recently employed been used as vaccines to fight against COVID-19. Ad is generated by transfection of human embryonic kidney (HEK) 293 or PER.C6 virus producer cells with plasmid vectors or by infection with replication defective Ad stocks obtained from crude Ad containing cell lysates to amplify the attenuated vector particles. These can be subjected to CsCl density gradient ultracentrifugation or chromatographic methods in the downstream processing of pure, high virus titre. Ad stocks from cell lysates are impure and can often result in reduced gene transfer and particle yield and column blockage during the purification process. As an alternative way to rapidly generate vastly cleaner virus working stocks for characterisation to high quality master stocks, we explored aqueous-two phase systems (ATPS). After testing multiple ATPS formulations, 20% - 20% w/w PEG 600 - $(NH_4)_2SO_4$ was found as most effective for Ad partitioning with up to 97(+/-3)% yield for high titre virus, devoid of aggregates that was effective *in vitro* and *in vivo* without observable toxicity. Importantly, particles stored frozen or at 4^oC show negligible loss of titre and are ideal for downstream processing to clinical grade to support the growing need for Ad vaccine production.

P0824

At-scale autologous CD19-CAR T manufacturing from whole blood collection for the treatment of autoimmune disease: process and product quality assessment

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Chimeric antigen receptor T (CART) cells targeting B-cells have been approved for treating various cancers and have now also demonstrated efficacy in refractory autoimmune diseases, including, systemic lupus erythematosus, myositis, and systemic sclerosis. Despite the efficacy of CAR T cells in autoimmune diseases and cancer, significant logistical barriers exist, limiting the availability of this therapy to patients. Access to apheresis centers is one of these obstacles, as patients need to undergo apheresis to isolate T-cells for CAR T cell manufacture. Therefore, we have developed a novel approach to manufacture CAR T cells without the need for patients to undergo apheresis. Whole blood, collected from patients utilizing a method similar to blood

donation, was used to isolate, transduce, and expand T-cells for the generation of CD19-CAR T cells.

Whole blood derived CAR T cells were compared to apheresis derived CAR T cells. Small scale manufacturing runs demonstrated that whole blood derived CD19-CAR T cells were similar in transduction efficiency, phenotype, cytolytic activity, and proliferative capacity to apheresis derived CD19-CAR T cells (Table 1). In addition, at-scale manufacturing runs were performed, which showed that product quality data were consistent to historical at-scale development batches (Table 2). Further, strategies for comparability assessment, including starting material and final product quality attributes, and implementation are being developed. Taken together, our data support the ability of whole blood derived T cells for manufacturing CD19-CAR T cells, which may address some of the logistical challenges to patients and enable broader access to CAR T cell therapies.

Table 1: Small scale CD19 CAR T runs using whole blood and leukapheresis from paired healthy donors

Donor	%CD19 CAR+	Fold Expansion	Final CD19 CAR+ Cells
HD1 LUK	75.8	70.8	2.68E+8
HD1 WB	64.9	75.2	2.44E+8
HD2 LUK	82.1	73.2	3.00E+8
HD2 WB	69.9	67.2	2.35E+8
HD3 LUK	79.4	69.6	2.76E+8
HD3 WB	68.2	65.4	2.23E+8
HD= healthy donor, LUK= leukapheresis, WB= whole blood			

Table 2: Large scale CD19 CAR T runs using whole blood and leukapheresis from healthy donors

Donor	%CD19 CAR+	Fold Expansion	Final CD19 CAR+ Cells
HD4 WB	80.8	60.5	3.81E+9
HD5 WB	66.3	35.8	2.35E+9
HD6 LUK	84.7	41.3	3.50E+9
HD67 LUK	78.4	42.7	3.35E+9
HD= healthy donor, LUK= leukapheresis, WB= whole blood			

P0825

Evolving synthetic Membrane Associated Accessory Proteins (MAAPs) for enhanced AAV vector egress

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The infectious biology of Adeno-associated virus (AAV) has been widely studied with particular attention to receptors and intracellular trafficking; however, unanswered questions surrounding capsid assembly, genome packaging, and viral egress remain. Our lab previously demonstrated that the membrane associated accessory protein (MAAP) functions as a viral egress factor. Specifically, we observed the ability of MAAP proteins derived from different AAV serotypes to trans-complement AAV capsid secretion from HEK293 producer cells. In addition, different structural domains of MAAP were mapped to functional attributes such as expression, linking, and membrane binding. Here, we develop engineered systems to increase the abundance and quality of AAV particles secreted into cell culture supernatant to improve the upstream manufacturing process. In addition to increasing titer, this method may allow for continuous perfusion systems to bypass the need for lysing cells, minimizing host cell contaminants in purified viral preps. Specifically, we dissect the impact of temporally controlled MAAP expression on the functional dynamics of AAV particle secretion using inducible producer cell lines. Further, we evaluate the interplay between Adenoviral helper genes as well as AAV Rep/Cap expression and MAAP induction on secretion efficiency. To further understand the impact of different MAAP structural components, we generated targeted saturation libraries which were subject to evolutionary selection using an infectious AAV cycling approach. This strategy yielded a diverse collection of novel MAAP variants with markedly improved AAV secretion profiles. Further, our directed evolution helps establish structure-function correlates of natural and engineered MAAP domains that facilitate robust AAV egress. Together, these findings provide a roadmap for enhanced upstream manufacturing of recombinant AAV vectors at high yield and improved quality.

P0826

Impact of CPPs on NK cell CQAs to improve the understanding of cell expansion – an experimental study using design of experiments

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Human natural killer (NK) cells represent a promising leukocyte population to use for immune cell therapies. Solid, as well as hematologic, tumors have been shown to respond to treatment with autologous or allogeneic NK cells. Providing NK cells in sufficient quantity and quality (i.e. efficacy, safety, identity, purity and yield), still remains a challenge. A deeper insight into the critical process parameters (CPP) and critical quality attributes (CQA) of primary NK cell culture is needed for the understanding and control of NK cell manufacturing. Due to the large number of CPPs, a design of experiments (DoE) approach was used – instead of the traditional trial-and-error or one-factor-at-a-time methods – for the systematic planning and for the reduction of the

number of experiments. Primary NK cells were cultivated under batch conditions, with varying CPPs such as glucose, glutamine, serine, asparagine, pyruvate, over a time course of twelve days and harvested every other day. Cytotoxic functionality was determined in co-cultures with K562 cells at every harvesting point. Additional cell culture parameters, several metabolites and cytokines of the single culture and co-culture were measured at every harvest day, while oxygen concentration as well as the pH of the single culture were monitored continuously. CQAs, such as the viable cell count, purity, lineage markers, activating receptors and death ligands, were investigated using flow cytometry. A detailed analysis of the impact of the investigated CPPs in the DoE was conducted and the DoE based experimental setup allowed the interpretation of the influence of each selected process parameter as well as their combination on the CQAs relevant for NK cell manufacture. In further steps of the project, selected CPPs will be used to formulate a data-driven model for a better representation of the primary NK cells' behavior. The aim of the model is to deliver further insights for process understanding and will be validated by future experiments, with the perspective of achieving a digital twin of the NK cell culture.

GM & CS have shared first authorship.

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P0827

Viral vector capture using membrane chromatography: Boosting particle recovery and infective yield through matrix design and optimized binding strength

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Viral vectors are the most widely used tools for the transfer of genetic information in gene therapy applications. The rapidly growing number and advanced state of clinical studies in this field require optimised processes for the efficient purification of these biopharmaceuticals. High functional yield and purity are critical process parameters.

Due to their size, typically in the range of 20 to 300 nm, viral vectors show hardly any diffusive behaviour in solution, but at the same time are usually extremely sensitive to a variety of process parameters of all kinds. Convective stationary phases address these challenging boundary conditions to a high degree. Their binding capacity, which is almost independent of the residence time, enables the binding of the target compound almost independently of the flow rate and thus enables high productivity with minimal exposure to adverse process-related conditions.

A cellulose membrane platform (Sartobind Convec) was developed specifically for binding and elution of viral vectors, which is characterized by a hydrophilic backbone with minimal non-specific protein binding, high homogeneity and high permeability.

First, the cellulose scaffold was modified with sulphate ligands to generate a binding surface similar to a mucosal cell surface. The resulting pseudo-affinity ligand enables the binding of e.g. influenza viruses with high binding capacity and selectivity without compromising product recovery. The performance of the sulphated cellulose adsorber is evaluated in comparison to

commercially available sulphated carbohydrate resins with three different influenza virus strains produced in a cell culture.

Amongst the viral vectors, lentiviruses stand out for their particular sensitivity. The modification of the membrane surface with anion exchanger functionalities and the adaptation of the binding strength yielded an AEX membrane with unprecedented lentivirus recoveries of up to ~70%. High process recoveries were obtained for two separate LV constructs encoding a GFP marker gene and a therapeutic CAR transgene. The membrane proved sufficiently permeable to allow the assembly of scalable devices from 1-150 ml bed volume with up to 8 mm bed height, showing no notable sign of pressure increase even after loading more than 400 membrane volumes (MVs) of clarified, but otherwise untreated cell supernatant. Running at 10 MV/min at moderate pressure, process times could be minimized to keep the exposure of the target to challenging conditions short. Sharp and concentrated product fractions were successfully eluted at comparably low ionic strength. Interestingly, most proteinaceous and DNA contaminates were found to pass through the adsorber during the loading phase, thereby simplifying the separation problem between impurity and virus considerably.

The unique capabilities of the membrane-based stationary phases presented are expected to significantly increase productivity associated with the capture step in the purification of virus particles. Due to their ease of use, sterilizability with gamma radiation and single (batch) use in closed processes, they represent a major step forward in the development of an optimized and efficient platform technology for the viral vector industry.

P0828

Optimization of automated selection and lentiviral vector mediated transduction of MSCs

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Mesenchymal stem cells (MSCs) are adult stem cells that can be isolated from several adult tissues and are of great interest for the development of new MSC-based products in advanced therapies or regenerative medicine. They have a wide range of therapeutic applications and are a valuable tool to treat different pathologies.

MSCs have traditionally been isolated by manual flask-based methods based on their property of adherence to plastic. However, this classic approach includes many open handling steps that can be error prone and labor intensive. Automation has the potential to address these problems and so, aiming to an industrial translation of cell and gene therapies, we investigated Quantum cell expansion system (Terumo BCT) for isolation and first cultivation passages of MSCs from unprocessed bone marrow (BM). The applied system is a hollow fiber bioreactor with a 1.7-m^2 surface area for cell growth and continuous medium perfusion. Optimal yeald were confirmed to be achieved (range 49-98x10^6 recovered MSCs starting from 50-100ml of BM aspirate).

In addition, in the context of product early stage development, we demonstrated the feasibility of lentiviral vector-genetically modified MSCs production to generate cell-therapy-based drugs or for downstream application as exosomes production.

Lentiviral vectors (LVV) are a platform to deliver and permanently express therapeutic genes and have displayed great potentiality in clinical trials.

Here we present proof of concept results concerning the setting of optimized MSCs transduction protocols (both in 2D and 3D systems) with an highly-purified VSV-G pseudotyped LVV encoding for GFP protein in order to obtain efficient genetic modification and process yield. Briefly, as regards 2D protocol in open setting, BM donor-derived MSCs were cultured in different chemically defined MSC-specific serum free media and seeded at different concentrations in tissue colture supports. Transduction was performed at different timing up to 24 hours from seeding at MOI ranging from 20 to 50. Different conditions were tested and the final selected protocol allowed to obtain optimal transduction efficiency (up to 80% GFP positive cells) by preserving critical functional attributes of MSCs. In parallel, an innovative automated transduction protocol in hollow-fiber bioreactor (Quantum cell expansion system) was designed. Transduction was performed using cryopreserved BM derived-MSCs as a starting material and pre-expanding them in order to reach optimal confluence level prior to the transduction step. The cells were transduced on day 4 with the same GFP LVV previously described at MOI ranging from 25 to 50 and further expanded until day 6. Optimal genetic modification results were confirmed to be achieved also in closed system (up to 86% GFP positive cells).

P0829

Scalable and cost-effective cell & gene therapy process development via multi-objective optimization

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The viability of cell and gene therapies (CGT) as a sustainable healthcare solution is challenged by their high costs, with factors such as expensive materials, stringent quality control measures, and the need for cleanroom facilities contributing to the multi-million pound price tags. Investment in developing scalable and cost-efficient processes at the pre-clinical stage can address some of these challenges but is often disincentivised by process complexity or time and budget constraints.

Digital twins represent a cost and time-effective solution for improving process development. By using mathematical models, many process alternatives can be tested efficiently to navigate complex process development trade-offs. In most cases, multiple competing objectives—such as productivity and cost—need to be balanced. Multi-objective optimisation (MOO) methods help to navigate these trade-offs by characterising the optimal balance between objectives in the form of a pareto front. A caveat for these methods is that they typically require thousands of process evaluations, which is infeasible in vitro, and thus require the use of an in silico model or digital twin. While the development of these models and execution of a MOO strategy requires a specific skill set, the end result—the pareto front—can readily be used by process developers without specialised expertise.

Our study presents a novel application of MOO for optimising the process length and refreshment strategy of a human mesenchymal stem cell expansion process in a fixed-bed bioreactor, focusing on yield, throughput, and cost-efficiency as process objectives. For modelling the cell expansion, a hybrid model was used. Hybrid models are an attractive compromise between the advantages of purely data-based models (fast development times) and mechanistic models (process insight, model generalisation). Moreover, the hybrid model developed only uses

local information and thus can be considered scale-independent. The cell expansion model was combined with a cost-of-goods model to calculate the costs of running a process, including materials, labour, and overhead (machines and facilities) expenditures.

The combined model was then used to perform MOO of the cell expansion process. The results illustrated that the throughput of the process could be improved by 39.6%—from 4.6 to 7.7 billion cells annually—while maintaining the same cost-efficiency. Alternatively, the cost-efficiency could be improved by 19.3%—from 133 to 164 million cells per €1,000—while maintaining the same yield. Moreover, it was also shown that the pareto fronts capture delicate nuances in the optimisation of the medium refreshment strategy, which offers increased process understanding. Experimental validation of these two optimised processes confirmed the model's accuracy, although some discrepancies highlighted the need for potential fine-tuning.

In conclusion, our MOO approach offers a cost-effective and fast method for optimising CGT manufacturing processes, while balancing multiple objectives, which is difficult or nearly impossible to achieve through wet-lab experimentation. The use of pareto fronts simplifies the decision-making process, making advanced optimisation accessible even to those without specialised expertise in statistical or process modelling. Antleron is one of the first companies to demonstrate these capabilities in the CGT space.

P0830

Generation of a stable high-titer production cell line for therapeutic AAV vectors

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Adeno-associated virus (AAV) vector based gene transfer is becoming routine in gene therapy. To meet the growing material demands, the use of stable cell lines for AAV-vector production is gaining more and more attention in industry and science. Major advantages are scalability and high reproducibility paired with lower cost of goods compared to triple-transfection based processes.

In a close collaboration between Roche, Spark and Cytiva (formerly Cevec Pharmaceuticals) we run a proof-of-concept study, which aimed to demonstrate the utility and effectiveness of the ELEVECTA™ technology for the generation of stable high-titer producer cell lines for the manufacturing of therapeutic AAV-vectors. In this case study we used a non-clinical variant of the alpha-Glucosidase (GAA) gene as clinically relevant transgene in combination with an engineered capsid.

We will present the workflow for the generation of stable AAV-vector packaging and producer pools and subsequently the generation of single cell clones. We will further show pool and clone characterization data in deep-well-plates and Ambr15 micro-bioreactors, the results of a stability study covering the critical time frame from cell banking to large scale production runs for selected clones as well as the results from mid-scale bioreactors for 2 final clones.

P0831

Improved DNA removal in the downstream processing of enveloped virus-like particles (VLPs)

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The development of biotherapeutic applications for vaccines and gene therapies is growing rapidly. To this end, enveloped virus-like particles (VLPs) and viral vectors are being used in various applications.

Many enveloped virus-derived particles reveal a slightly acidic isoelectric point. Due to their high sensitivity to pH values deviating from neutral, chromatographic separation methods based on anion exchange chromatography (AEX) are particularly suitable for the purification of these particulates. In particular, convective stationary phases allow high process speeds and thus extremely gentle handling of this class of biopharmaceuticals. Contamination with other anionic cell components, in particular DNA, chromatin complexes and extracellular vesicles (EVs), has proven to be an inherent problem in the chromatographic purification of viral particles using AEX. The FDA has set particularly stringent guidelines for the depletion of DNA contaminants at 10 ng per dose and a fragment length of less than 200 bp.

HIV-VLPs have attracted increasing attention as a promising platform for the production of vector vaccines against HIV. However, due to their convenient handling and modifiability, they can be used as model systems for lentiviruses. In this study, we use fluorescently labeled HIV-VLPs produced by a HEK 293 cell line to optimise the downstream processing for enveloped virus particles. The fluorescent label allows the rapid detection of VLPs and differentiation to other particulate contaminants like EVs.

First, we monitored the release of nucleic acid contaminants during VLP upstream processing. Using the clarified harvest, DNA digest conditions with several nucleases were optimised and degradation products were characterised. The clarified harvest is used to optimise DNA digestion conditions with multiple nucleases and to characterise degradation products. In the next step, the separation of the desired VLP constructs from other cellular components utilizing AEX membrane adsorbers was investigated.

As a result, we present a comprehensive qualitative and quantitative analysis of the degradation products of cellular DNA contaminants and characterise their chromatographic separation behaviour. We expect that our data will provide substantial guidance for the design of DNA digestion and chromatographic depletion of chromatin fragments. We anticipate that the findings presented here will also foster future studies aiming at the optimization of purification strategies for enveloped viral vectors reaching high purity and minimizing product loss.

P0832

Harnessing the power of High-Throughput Virology and Design of Experiment for rapid vector production process optimization

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Biomanufacturing of cell and gene therapies is a complex process, hampered by variability in yield, quality, and scalability of manufacturing platforms. Production parameters can be key drivers for vector quality, quantity and cost, making them prime process optimization targets. However, assessing all possible variables and understanding their interactions with product output and each other can be a convoluted and time-intensive task when using traditional one-factor-at-a-time (OFAT) process optimization strategies.

A high-throughput, 96-well format virology platform using transduction-based quantification of luciferase reporters allows for rapid quantification of various produced viral vectors. We developed transfection-based production and quantification assays for both AAV (suspension 293) and lentivirus (adherent 293T) and used DoE statistical methods to optimize up to six different production parameters simultaneously. This approach of combining high-throughput virology assays with DoE is amenable to various vector production platforms, including replicating viruses, reverse genetics and inducible systems.

AAV production in suspension HEK293s was optimized, with the methodology allowing simultaneous optimization of up to six factors, including total DNA, nucleic acid type, cell count, transfection reagents, and addition of small molecule enhancers for optimization of AAV production. Additionally, LV production optimization in adherent HEK293T by full factorial combination of various plasmid ratios was performed. Importantly, the optimized conditions identified at a small scale were translatable to larger-scale formats, demonstrating scalability.

We demonstrate that combining high-throughput assays with the statistical power of DoE enables process optimization of multiple factors in time-frames not achievable through traditional OFAT methods. Furthermore, we exemplify how these methods can be used to optimize cell and gene therapy manufacturing processes for various factors including transduction efficiency, TU/mL, vg/mL or even cost/vg. The flexibility of optimizing different outputs from a single data set unlocks the potential for data-driven decisions to maximize production processes based on strategic needs.

P0833

Novel 'ultra-short' liquid chromatography columns for efficient high-throughput oligonucleotide analysis

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The number and variety of cell and gene therapy products is growing rapidly. Importantly, these new drug candidates are becoming increasingly complex, whether in terms of increasingly complex molecular composition or mode of action. More than ever, the industry needs new analytical methods to characterize, formulate, and perform release testing on these new drugs. Increasing the throughput and amount of data generated by an analytical method will help bring more effective drugs to market faster.

Among analytical techniques, liquid chromatography separations offer several capabilities and advantages. The presentation focuses on reversed-phase (RP) separations, but the same rules apply to any retentive chromatography mode for large molecule analytes.

We show that very short columns (a few millimeters to centimeters) are required to effectively retain and separate large molecule analytes. The recently introduced "ultra-short column" approach allows complex separations to be performed in 0.5 - 3 minute intervals. (Traditionally, such separations are performed at 10 - 60 minute intervals using 15 cm columns.) The ultra-short column approach can dramatically reduce method development time. The column hardware is specially designed to mitigate unwanted and non-specific interactions. The inert hardware is based on a hybrid organic-inorganic silica material. The use of this low-adsorption material improves the sensitivity and reproducibility of the measurements and enhances productivity as well.

Some generic (platform) gradient approaches are also discussed, based on the consideration of the so-called "homologue rule". Various examples are shown to illustrate the potential of ultra-short columns and some novel gradient approaches.

Ultra-short columns are probably the most beneficial for high throughput analysis for manufacturing and bioanalytical laboratories that need to analyze 100-1000 samples on a regular basis.

P0834

TESSA® Platform for Transforming Scalable and Versatile Recombinant AAV Gene Therapy Production

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The efficient manufacturing of recombinant AAV to meet pre-clinical and clinical demands remains a significant challenge for AAV gene therapy. To address this challenge, we have developed a novel self-silencing helper adenoviral vector system, termed TESSA®, genetically modified to self-inhibit expression of its late adenoviral structural proteins, while delivering all the necessary components for the efficient and contaminant-free manufacture of AAV in serum-free suspension HEK293 cells.

TESSA® vectors are engineered to express AAV rep and cap genes stably, and to deliver the AAV GOI transfer genome. They are based on E1/E3-deleted adenovirus type 5 vectors modified with Tet repressor binding site inserted into the Major Late Promoter (MLP), which drives adenoviral structural protein expression, while the TetR gene itself is expressed from the MLP. This genetic modification creates a negative feedback loop, where adenoviral structural proteins can only be

produced in the presence of doxycycline, but allow AAV production in the absence of doxycycline.

TESSA® serves as a robust and versatile platform for producing AAV, offering flexibility through two distinct approaches. The process variation between the two models differs by the approach in which the gene of interest (GOI) is introduced into the HEK293 cells. A TESSA® vector encoding Rep and Cap can be combined with either a TESSA® vector (TESSA® Duo model) or an AAV (TESSA® Pro model) to deliver the AAV GOI transfer genome for AAV production.

While the TESSA® Duo requires the construction of two TESSA® viral banks (TESSA® Rep Cap & TESSA® GOI), this process is highly efficient and capable of yielding over 30 times more AAV compared to the triple transfection process across various serotypes, including AAV1-9 & rh10, with productivities exceeding 1E+12 vector genomes/mL and >1E+6 VG/cell. We demonstrate that the TESSA® Duo process can be easily scaled up to 50L and 200L bioreactors for the production of AAV2 and AAV6 vectors and is capable of yielding >7E+11 GC/ mL of cell culture and final drug substances of >1E+17 GC / 200L.

The TESSA® Pro model enables a streamlined approach for AAV manufacture by amplification of AAV seed stocks using TESSA® RepCap. AAV master viral banks can be generated by the triple-transfection process and co-infection of HEK293 cells using TESSA® RepCap enabling >5,000-fold amplification of input AAV materials and yielding up to 2E+12 GC/mL of cell culture. Additionally, we observed significantly high efficiency for AAV packaging using the TESSA® Pro process, with up to 60-80% full capsid, by analytical ultracentrifugation (AUC), after affinity chromatography and enriched to 96-99% after polishing. This process is presumed to expedite the replication and packaging of the single-stranded rAAV genome post-nuclear entry, bypassing the conventional requirement for rescuing the AAV ITR embedded within the double-stranded DNA structure associated with other rAAV manufacturing systems.

TESSA® marks a major breakthrough in tackling the challenges of AAV vector manufacturing, delivering a scalable, efficient, and contaminant-free solution for the production of clinical-grade AAV gene therapy products.

P0835

Engineering novel binders for AAV purification using iterative phage display and machine learning

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Currently approved adeno-associated virus (AAV) based gene therapies utilize different capsid serotypes (AAV2/5/9/rh74). Downstream purification strategies for these capsids utilize a combination of ultracentrifugation, ion exchange and/or affinity chromatography. For affinity chromatography in particular, nano/antibody-based affinity resins such as CaptoAVB and CaptureSelect AAVX/8/9 are utilized to purify AAV for pre-clinical and clinical scale. However, challenges remain, such as the need for universal resins that can purify different natural AAV isolates as well as next generation engineered capsids. Additionally, there is a need to develop affinity resins that are amenable to milder elution conditions compared to commercial options, which minimally affect capsid integrity, while maximizing removal of host producer cell impurities. Here we describe the engineering of novel AAV binders using iterative phage display and machine learning techniques. Binders selected through this approach demonstrate both serotype-specific

and pan-AAV capsid binding capabilities at neutral pH, and robust elution under mild buffer conditions, in contrast to current commercially available affinity resins. Importantly, negative selection of the library on HEK293 lysate reduced non-specific binding and enabled efficient removal of host cell protein contaminants. This new class of AAV capsid binders offers a promising improvement to current downstream purification methods that can ensure AAV vector quality and safety.

P0836

Development of a high yield/high purity MSC-EV downstream process that maintains EV CQAs

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The number of clinical trials investigating extracellular vesicles (EVs) derived from mesenchymal stromal cells (MSCs) as a therapeutic agent has rapidly increased in recent years. However, efficient and clinically translatable methods to purify the EVs are still considered as one of the main challenges during processing. Cumulative post-purification yields of MSC-EVs are often <10%, representing another major challenge in total EVs per lot and high cost of goods. RoosterBio collaborated with Cytiva to identify critical process parameters to maximize EV yield and purity during downstream processing.

EV conditioned media was generated using hBM-MSCs, RoosterNourish™-MSC-XF expansion medium and RoosterCollect-EV medium across three 3L bioreactor runs as described previously. Conditioned media was then processed with or without Agent V treatment (RoosterBio, patent pending), followed by screening of different clarification filters. A flux excursion study was performed to identify optimal TFF parameters to concentrate clarified media. Concentrated conditioned media was used to evaluate different chromatography resins (Capto Core 400 and superSEC columns, Cytiva). Zetaview (Particle Metrix) and Bradford assay were used to measure particle concentration and protein concentration, respectively at each unit operation to report EV recovery and EV purity (particles/mg of protein). Jess automated western blot (Bio-techne) and CD73 activity assay were used to assess EV identity and bioactivity, respectively.

Treatment of EV conditioned media with Agent V followed by a filter train consisting of Profile II and Fluorodyne II DBL filters was optimal to reduce media turbidity (>50%) while maintaining high EV yield (≥70%). Agent V helped in alleviating pressure increase during filtration facilitating a 10-fold increase in throughput of 120 L/m^2 with agent V vs. 12 L/m^2 without Agent V. This consequently led to high EV recovery of ≥70% compared to 20% without Agent V. Clarified conditioned media was then concentrated 10-fold by volume through TFF using optimal transmembrane pressure and shear identified by the flux excursion study. Two commercially available chromatography resins were evaluated: multimodal ligands with size-exclusion (Capto Core 400) and an EV-specific size exclusion-based resin (superSEC resin). For both resins, there was minimal pre-column pressure rise during the process, indicating adequate loading of the columns. Both resins achieved high EV recovery (>85%) and high protein impurity clearance, leading to an increase in purity level (>1E11 particles/mg of protein) compared to initial harvest (5E10 particles/mg of protein). EVs generated in this process, with either resin, maintained critical quality attributes (CQAs) of EV identity (expression of EV specific tetraspanins CD63, CD9 and CD81) as well as maintaining potency as indicated by presence of CD73 activity.

In this work, we generated a high concentration of clinically relevant EVs and identified critical process parameters for downstream EV purification. The developed process demonstrated high EV recovery with increase in purity while maintaining EV critical quality attributes. This study shows promising results of identifying an EV purification platform that can be tailored for a wide variety of therapeutic EV programs.

P0837

Engineering a competitive interaction between protein dimers for biomolecular circuits

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Biological circuits hold promise for revolutionizing gene therapy by providing precise control over therapeutic gene expression. However, the application of biological circuits to therapy is difficult due to several limitations: The size of circuits is usually too large to package them in therapeutic vectors, and there aren't many biological regulators we can use in circuits in mammalian cells other than Cas proteins. These proteins are large, can have off-target effects, and are immunogenic, making them unsuitable for use in therapy. In this study, we aimed to overcome these limitations by developing a compact, tunable, modular, and orthogonal regulatory tool for use in therapeutic biological circuits. Specifically, we focused on engineering a competitive interaction between protein dimers to modulate transcriptional activity in mammalian cells. We began by modifying a transcription factor that homodimerizes, reducing its dimerization domain, and introducing a synthetic coiled coil. We created the TF inhibitor by removing the DNA-binding domain from the monomer and adding the complementary coiled coil. This design promotes non-functional heterodimer formation, effectively inhibiting transcriptional activity. Experimental validation in HEK293T cells demonstrated an almost fifty-fold reduction in transcriptional activity. Furthermore, our results demonstrated the versatility of the system by showing its compatibility with a different DNA binding domain from human transcription factors. Finally, our findings indicate that this regulatory system enables the creation of smaller, compact biomolecular circuits, overcoming previous size constraints associated with gene therapy vectors. Thus, our study presents a promising approach for advancing the application of biological circuits in gene therapy.

P0838

Engineering an enhanced rAAV6 manufacturing process resulting in higher yield and quality

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In recent years, rAAVs have been gaining popularity in the development of cellular immunotherapies, and among the rAAV serotypes currently in-use for this purpose rAAV6 has shown the highest efficiency to transduce T cells. Even still, a high concentration of viral genomes per cell is required. In order to meet the growing demand for these vectors, we have developed an enhanced process for rAAV6 production.

Here we demonstrate a multi-pronged process development approach, resulting in rAAV6 production at a concentration >5x1011 GC/mL and achieving a full capsid percentage of greater than 45% at harvest, some of the highest yields reported to date. and an oversized client construct as our transgenes, various upstream parameters including cell density at transfection and transfection enhancer concentration were evaluated to determine their effect on rAAV6 production. The design of experiments (DOE) methodology was also employed to identify the optimal plasmid ratio for transfection, and plasmid engineering strategies were undertaken to further improve rAAV6 production, both of which resulted in further enhancement of rAAV yield and quality. The scalability of our optimized process was demonstrated using a benchtop single-use bioreactor system from Eppendorf. To determine the success of these optimization strategies, genome copy titer was measured via digital droplet PCR (ddPCR) with specific gene-of-interest primers on the QX600 ddPCR system (Bio-Rad). Further product quality characterization of the rAAV6 produced by our process included measurements of contaminating host-cell DNA by ddPCR, host-cell protein by ELISA, and genome integrity by NGS.

The development of this robust production process offers a promising approach to enhance rAAV6 manufacturability, offering greater than 4-fold improvement in genome copy titer and 3-fold improvement in full capsid percentage at harvest over our baseline. These improvements will have an immediate impact on reducing the cost per dose, helping to accelerate the development of valuable gene and cell therapies using rAAV6 vectors. In summary, this work demonstrates our ability to efficiently increase rAAV6 packaging efficiency and yield via optimization of upstream process parameters, an approach which is easily transferrable across rAAV serotype and gene-of-interest.

P0839

Development Challenges of AAV and LVV Manufacturing Processes: A Comparative Perspective

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AGC biologics is a leading global CDMO, providing world-class development and manufacture of mammalian and microbial-based therapeutic proteins, plasmid DNA, viral vectors and genetically engineered cells.

Adeno Associate Vectors (AAV) and Lentiviral vectors (LVV), produced with transient transfection in HEK293 and HEK293T cells are extensively used for genetic modification in gene therapy for *ex-vivo* and *in-vivo* treatments.

AGC Biologics developed BravoAAV and ProntoLVV platforms for the manufacturing of AAV and LVV at different scales in adhesion and suspension. Both platforms are flexible to be adapted to different GOI (Gene Of Interest). Of note, BravoAAV positively applies to different AAV serotypes.

AAV and LVV share some characteristics but are different from many points of view. These resulted in the development of dedicated manufacturing platforms even if starting from common process steps using same equipment and common set of reagents but that faced different challenges.

We are going to compare the two manufacturing processes, step by step, underlining differences and common features.

Packaging cells HEK 293T and HEK 293 cells are used for LVV and AAV production respectively. It has to be taken into account that those cells have different behavior in terms of growth rate and aggregation profile.

Transient transfection is of course performed with dedicated plasmids, but with the same transfection reagent.

The strong proteic capsid of AAV ensures their higher resistance to shear stress, allowing to perform harvest later than for LVV and also HEK293 cell lysis.

On the contrary, the presence of the envelope in LVV is the cause of the high sensitivity of the vector to temperature, pH and shear stress. For this reason, clarification of LVV produced in suspension is challenging, as the whole cell mass have to be separated from the supernatant.

Historically, the purification of LVV has primarily relied on AEX Chromatography, whereas for AAV, a wide range of affinity resins is available on the market.

During the execution of concentration and diafiltration steps, AAV is more resistant to shear stress and high recoveries of infective vector are more easily achieved. In contrast, LVVs require more gentle conditions and are also susceptible to aggregation phenomena due to the stickiness of their envelopes, negatively affecting the last critical step: sterilizing filtration.

Sterilizing filtration is performed with 0.22 µm filters for both platforms. Since LVV diameter is approximately 0.10 µm, the presence of vector aggregates can cause filter clogging and a decrease in recovery. The yield of this step for LVV is around 40-50%. Due to their compact size and reduced inclination to aggregate, AAVs demonstrate a more straightforward behavior during sterilizing filtration. These characteristics prevent membrane clogging and lead to higher recovery rates in the process, up to 90%.

In the analytical panel there are some assays that are shared by the products and others that are specific and dedicated.

The knowledge of the biology of AAV and LVV and the implications in the manufacturing process and assay development is a key factor for the flexibility, capacity and capability of a CDMO.

P0840

Endonuclease Treatment in Downstream Processing of Virus-Like Particles

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In the production and purification of bio-nanoparticles (BNPs) such as viruses, viral vectors, and virus-like particles (VLPs), it is essential to remove host cell DNA, which is a major impurity. Regulatory agencies like the FDA and EMA require that residual host cell DNA in injectable drugs be less than 10 ng per dose. Removing DNA from BNP products is particularly challenging when it

is present in the form of chromatin. Chromatin consists of DNA wrapped around histone proteins, forming structures that are similar to BNPs in size and surface charge, making separation difficult without disrupting the chromatin. Endonuclease treatment is a key step in manufacturing viral vaccines and gene therapies to reduce host cell DNA content. However, tightly packed DNA in the form of chromatin is not accessible for digestion using conventional endonucleases. In the past, chromatin has often been overlooked due to the lack of a clear distinction between free DNA and DNA in the form of chromatin and lack of proper analytical methods for chromatin detection and quantification. Traditional endonucleases are not capable of removing DNA in the form of chromatin. One way to address this issue is to use salt-active nucleases and increased ionic strength. Endonuclease treatment can be applied at various stages of the process, depending on the selected unit operations and the impact of DNA content on their performance.

In this study, HIV-1 Gag-based VLPs produced in HEK-293 cells were used as a model BNP. The downstream process train following the harvest included flowthrough and affinity chromatography. For the endonuclease treatment step, two different endonucleases were investigated: Benzonase, used as the benchmark, and M-SAN HQ, a salt-active endonuclease. It was shown previously that M-SAN HQ is capable of digesting chromatin. Additionally, the placement of endonuclease treatment in the downstream process train was investigated. Multiple scenarios were tested, each varying the point at which the endonuclease treatment was applied. The results from each scenario were analyzed to identify the most effective stage in the process for reducing DNA content in the final product.

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P0841

Leveraging Platform and Process Characterization Data to Accelerate CGT Validation and Commercialization

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As of early 2023, almost 30 different cell and gene therapies (CGTs), with diverse delivery vehicles and routes of administration, have been approved by regulatory authorities to address a variety of diseases. Many more products are in development and the number is expected to grow exponentially in the coming years. The CGT industry faces many challenges, including restricted availability of material for development and clinical use, limited product understanding and analytics, limited prior knowledge and high cost of goods, among other concerns. To address some of these, there is a strong desire to create common approaches to manufacture novel therapies, which in some cases only differ by their genetic payload. Such ‘platforming’ approaches require a good understanding of regulatory requirements, manufacturing facilities and equipment design, production process design as well as process parameters (and their potential impact on product quality) across therapeutic technology platforms. Despite interest in such approaches, and some initiatives in this area, there is currently no available guidance or best practice to aid CGT developers in this regard. To fill this gap, CGT companies from BioPhorum have evaluated

and summarized opportunities to leverage process development and characterization knowledge across similar therapeutic technology platforms to accelerate subsequent process validation activities using experience gained with similar product types. These examples and approaches are informed by the results of a survey of 20 member companies within BioPhorum CGT. We believe this initiative will be a valuable resource to CGT developers as the industry strives to move forward with such platforming and prior-knowledge-based approaches, which are already well established for other therapeutic modalities.

P0842

An enhanced protein language model for cell surface protein identification in genomic therapies

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1: WhiteLab Genomics

Genomic therapies promise durable cures for previously untreatable diseases by enabling precise targeting of affected tissues or cells. Advances in target specificity, such as designing binders through rational or guided strategies, have significantly improved efficacy. However, experimental validation remains prohibitively expensive. Identifying specific cell surface receptors is crucial for increasing the transduction efficiency of target cells. This step enhances efficacy, reduces immunogenicity, and prevents off-target transduction, thereby minimizing unwanted effects. Our objective is to accelerate the research and development of gene therapy vectors by optimizing the identification of target-specific receptors, thereby limiting the number of potential candidates, and reducing associated costs.

To address this need, we developed a deep learning method capable of predicting whether a protein is located at the cell surface, using only its amino acid sequence, and applicable to proteins of any length. Numerous surfaceomes—databases cataloguing cell surface proteins—have been established through various means, including experimental methods, computational approaches, and hybrid techniques. Existing methods often depend on multiple pre-computed features or focus on predicting other topological domains rather than specifically identifying surface proteins. However, a consistent and reliable gold standard is currently unavailable, and these databases represent limited sets, restricting their scalability. Our strategy incorporates biological context, a rigorously curated training dataset, and advanced deep learning techniques, eliminating the need for feature selection and thereby enhancing the model's generalizability.

Our initial model, which demonstrated 84% accuracy in distinguishing surface from non-surface proteins, was presented at the ASGCT congress. Building on this success, we have refined our model with several key improvements. We constructed different training datasets filtered by taxonomic ranks (eukaryota, metazoa, or mammalia) and customized the balance between positive and negative observations. The test dataset, composed exclusively of human proteins, was derived from a portion of the training dataset used in the initial version, ensuring relevance to human genomic therapies.

Additionally, we introduced a custom sampling technique that, during each training epoch, selects a different protein from each cluster of proteins with similar identities. This approach effectively performs data augmentation, enhancing the robustness and diversity of the training data and ensuring that no single protein is overrepresented.

The updated model, still based on ESM-2, no longer relies on an ensemble approach. Instead, the best model from an extensive hyperparameter search has been selected, resulting in a streamlined and more efficient prediction process.

The model has been tested on a more realistic dataset with a distribution of 20% cell surface proteins and 80% non-cell surface proteins, reflecting real-world scenarios. This configuration has demonstrated improved performance and better generalization capabilities, particularly in specific configurations.

With these advancements, our model achieves robust performance in identifying cell surface proteins, contributing to the accelerated development of targeted therapies. This approach not only reduces experimental validation costs but also paves the way for the discovery of novel candidate receptors, driving forward the field of advanced therapeutics.

P0843

Considerations for biosafety testing of cell and gene therapies

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Regulatory authorities such as the US Food & Drug Administration (FDA) and the European Medicines Evaluation Agency (EMEA) impose stringent limits on the amount of microbial contaminants and impurities present during the manufacturing of biological medicines and vaccines, and present in cell and gene therapy products. These regulations ensure sterile products and thus patient safety. To establish that the testing procedures are accurate, regulatory authorities require proof of testing before clinical trials can be approved. Consequently, all components of the manufacturing process must undergo extensive safety testing to demonstrate identity, stability, and purity. This poster will review general approaches to biosafety testing, with specific focus related to cell (for example CAR-T cell therapies) and gene therapies.

Key Discussion Points:

- Cell bank & Virus Seed Biosafety & Characterisation, including a brief overview of
    - Identity Testing
    - Genetic Stability
    - Purity (freedom from bacteria, fungi and mycoplasma)
    - Virus Safety will be the focus of the talk, considering
        - Broad specificity approaches *in vitro*, *in vivo*, NGS non-targeted
        - Retroviruses – *infectivity /EM/RTase/PCR*
        - *Species specific - PCR / targeted NGS / 9CFR / MAP / HAP*
- How are viruses detected?
- Considerations for viral vectors

P0844

Boosting/Enabling closed Chromatography Processes using a Gamma Sterilized and Scalable Chromatography Product Platform from Small to Large Scale

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1: Sartorius Stedim Biotech

With an increasing number of cell and gene therapy trials on the horizon, the demand for cost-effective and high-quality manufacturing of gene delivery tools such as viral vectors or exosomes is growing. Although there is still little agreement on the optimal sequence of unit operations required for manufacturing high yields of vectors, chromatography, particularly anion exchange chromatography, is commonly used for primary capture from cell culture supernatant or polishing albeit with typically low recoveries or yields). Membrane chromatography has already proven to be an outstanding technology in terms of chromatographic performance recovery/yield and productivity in several processes.

This study illustrates the scalability of a cellulose-based membrane chromatography portfolio from development to manufacturing scale using both model and real-application feed streams. The portfolio includes strong (Q) anion, weak anion, and heparin functionalized membranes. Equipped with sterile connectors and tubing, the apparatus can be gamma irradiated within its shipping box, significantly reducing the bioburden risk and improving handling by enabling a closed aseptic process. The high permeability of the chromatographic membrane allows loading several hundred column (membrane) volumes without a significant pressure increase at a flow rate of 10 column volumes per minute thereby significantly reducing process times and minimizing the exposure time of the target to demanding process conditions.

An outlook is provided regarding the commercial availability of this novel and innovative membrane chromatography portfolio designed for single use, closed and aseptic processing, which aids in implementing cost-effective and high-quality manufacturing of gene delivery tools.

P0845

Thymidylate synthase disruption to limit cell proliferation in cell therapies

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Stem and progenitor cells hold great promise for regenerative medicine and gene therapy approaches. However, transplantations of living cells entail a fundamental risk of unwanted growth, potentially exacerbated by CRISPR-Cas9 or other genetic manipulations. Here, we describe a safety system to control cell proliferation while allowing robust and efficient cell manufacture, without any added genetic elements. Inactivating TYMS, a key nucleotide metabolism enzyme, in several cell lines resulted in cells that proliferate only when supplemented

with exogenous thymidine. Under supplementation, *TYMS*$^{-/-}$-pluripotent stem cells proliferate, produce teratomas and successfully differentiate into potentially therapeutic cell types such as pancreatic beta-cells. Our results suggest that supplementation with exogenous thymidine affects stem cell proliferation, but not the function of stem cell-derived cells. After differentiation, postmitotic cells do not require thymidine *in vitro* or *in vivo*, as shown by the production of functional human insulin in mice up to 5 months after implantation of stem-cell derived pancreatic tissue.

P0846

Enhanced AAV8 Capture Purification Using Sartobind Membrane Adsorbers: A Leap Forward in Gene Therapy Processing

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1: Sartorius Stedim Biotech GmbH 2: The Automation Partnership Ltd.

The field of gene therapy is advancing rapidly with the development of adeno-associated virus (AAV) vectors as the preferred delivery system for genetic material. Efficient and scalable purification of AAV vectors is critical for clinical and commercial success. This study focuses on optimizing Sartobind membrane adsorbers to capture AAV serotype 8 (AAV8), a serotype widely employed in gene therapy applications.

The study aimed to establish a membrane chromatography-based AAV capture process. Membrane chromatography has several advantages over conventional resins such as high flow rates, reduced processing times, easy scalability, reduced buffer consumption, no cleaning validation needed, and a fast adsorption and desorption process. In this study, we have developed a scalable capture purification protocol that significantly improves process performance.

The data presented herein details the optimization of the capture step using Sartobind S cation exchange membrane adsorber, starting with assessing the binding capacity at different pH levels. Subsequently, we improved the virus recovery and impurity removal during the Sartobind-based AAV8 capture step by conducting a Design of Experiments (DoE). This approach also aided in characterizing the influence of several factors on purification efficiency. Scale-up of the process (from a 1 mL to a 75 mL device) maintained high virus recovery rates and improved DNA reduction, confirming the scalability and robustness of the Sartobind technology for AAV8 purification.

The results of our study provide valuable insights into the use of Sartobind membrane adsorbers in gene therapy manufacturing. The optimized protocol provides a streamlined and effective solution for the capture of AAV8, with potential implications for other AAV serotypes. Our findings contribute to the advancement of gene therapy by ensuring the availability of high-quality AAV vectors for therapeutic use.

P0847

Automation preserves product consistency and quality for the formulation, fill, and finish of T cell-based therapies

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1: Terumo BCT Inc 2: Charles River Laboratories

As larger-scale allogeneic and increasing amounts of autologous cell therapies become available, manufacturing must keep pace. Controlling quality attributes like quantity, viability, sterility, and biological activity is crucial. Current cell therapy manufacturing is labor-intensive and time-consuming, with open-air operations increasing contamination risk and human error. Closing and automating processes can reduce risks, improve consistency, and lower costs, especially during final formulation and fill-finish steps.

In this study, the automated Finia™ Fill and Finish System (Terumo Blood and Cell Technologies, Lakewood, CO) was tested to efficiently automate the formulation and fill-finish of a T cell product. Finia is designed to fill up to four bags (three product + one quality) at a time, reducing hands-on and DMSO contact time during formulation. It was now investigated if this automated platform can be used to fill a larger number of cryogenic bags through an expanded process and whether the quality and uniformity of the cell product is maintained throughout the scaled-up process.

Commercially available T cells were activated, expanded for 12 days, washed, and concentrated prior to formulation, fill-finish, and cryopreservation. Finia was pre-programmed to use the Finia 50 disposable set and fill each drug bag to 20 mL and the quality bag to 10 mL with a final cell concentration of 13e6 cells/mL and final DMSO concentration of 5% at 15 °C. After four disposable tubing sets were processed, all product bags were transferred to a controlled-rate freezer and then stored in liquid nitrogen until post-thaw analysis.

This automated system was effectively scaled to four times its singular capacity in a two-hour time interval, with variation in cell number and product volume of less than 10% across all containers. Analysis of the different sub-lots of the final product revealed high cell viability and consistent T cell phenotype and low expression of T cell senescence and exhaustion markers. The functionality of the T cell product was compared by measuring cytokine response after restimulation, with secreted levels of effector cytokines like IFN-y and TNF-a being similar across the different sub-lots.

Collectively, these results show that automation can scale up the formulation and fill-finish process while maintaining cell product phenotype and functionality. Automation minimizes variability in cell health, phenotype, and functionality, ensuring precise dosing. Demonstrating an automated, scalable fill-finish approach that maintains product uniformity and quality is crucial for future cell therapies. This study investigated scaling the fill-finish process on Finia as part of the Charles River Laboratories and Terumo Blood and Cell Technologies collaboration.

P0849

Digital PCR applications for cell and gene therapy: High-quality detection of mycoplasma contamination

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Developing safe and effective biopharmaceuticals requires strict quality controls at all stages of the development process and during manufacturing. The rapid detection of potential impurities is crucial for ensuring high-quality advanced therapy medicinal products (ATMPs). Nucleic acid techniques (NATs) for mycoplasma contamination testing are well established and described by different pharmacopeias worldwide. Some compendia, including the European Pharmacopeia (EP), have announced a revision that will make the validation of NATs more stringent. Therefore, we present a workflow for detecting mycoplasma presence/absence based on a one-step RT-dPCR (reverse transcription digital PCR) to detect mycoplasma rRNA, as well as DNA. This allows for highly sensitive and robust contamination detection in a variety of sample matrices, such as media containing high salts or high amounts of producer cells. The validation report covering the complete workflow (and adhering to all the necessary criteria established by the guidelines) shows a limit of detection down to 5–10 CFU/mL for the most common mycoplasma species. Based on sequence alignment, at least 127 Mollicutes species can be detected. Furthermore, irreversibly inactivated and experimentally verified standards are crucial to establish and validate robust in-house NAT mycoplasma testing, while avoiding the handling of infectious mycoplasma. This need is met by the Mycoplasma Standard CFU Kits for all 10 compendia-mentioned species. The standard material allows the LOD verification at ≤10 CFU/mL and can also be used as positive control material.

P0850

Scalable fixed-bed technology enabling sustainable process development of HEK293-based viral vector upstream biomanufacturing

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With over 1400 clinical trials and more than 25 products with market approval, the cell and gene therapy (CGT) field is reaching a turning point. As the demand to treat larger patient populations and target diseases requiring systemic rather than locoregional delivery, the existing production methodologies yield insufficient functional viral vector particles (VVP), leaving many patients underserved. Radical rethinking of the production pipeline is key. Currently, commercial VVP production is based on HEK293 cells either grown in suspension or adherent on planar tissue culture plastic (TCP). However, TCP is not readily scalable, while suspension cell culture in bioreactors demands cellular adaptation and generates process complexities that affect downstream product quality and yield. Although fixed-bed bioreactors are used commercially for

adherent HEK293 scaling, they fall short when it comes to the fixed-bed spatial design, reproducibility, and customization. In addition, their scale and accompanying operational cost are a great barrier to adoption and optimization in academic and early biotech process development settings. CellGuide®, the proprietary smart fixed-bed bioreactor technology developed by Antleron, enables scalable adherent cell bioprocess development. The coupling of bespoke, controllable, and parallelized 3D fixed-beds with a digital twin and *in silico* experiments allows smarter and faster bioprocess development in small scale bioreactors (0.5 cm^3), with results being predictive of and translatable to a more production-relevant bioreactor scale (200 cm^3). This enables academic labs and start-ups to address process development questions early on, to have faster results (weeks instead of months) and to scale-up production to meet their needs, without skyrocketing costs. Here we report how CellGuide® was applied to translate a bespoke static, 2D TCP-based HEK293 methodology into a 3D scalable upstream perfusion bioprocess. Beta testing was performed at the Leuven Viral Vector Core (LVVC), an academic viral vector manufacturing platform. The technical and economic validity was assessed with reference cell lines and culture methods employed to produce VVP. The LVVC in-house virus like particles (VLP) and adeno-associated viral vectors (rAAV) production process, based on transfection in 2D TCP, was converted to a fixed-bed based perfusion process (0.5 cm^3) in a closed bioreactor. The functionality of the vectors was determined by assessing the payload delivery. The results demonstrate that the implementation is simple and effective, producing similar functional VLP titers as the 2D scenario even before conducting any optimization. Further research is ongoing to optimize the innovative production process using DoE and predictive computational modelling and to confirm scalability (up to 50 cm^3) of the process. The combined wet-lab and in-silico experimental results indicate that CellGuide® provides a closed solution for efficient producer cell supply (growth and harvest), efficient cell transfection and VVP harvest. Predictive analyses suggest that the translation would enable LVVC to produce up to 10x more functional VVP at an affordable cost within their existing lab and team capacity and speed up process development cycles from months to weeks.

P0851

Microfluidics for scalable culture of adherent cells

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Human induced pluripotent stem cells (iPSCs) and primary mesenchymal stem cells (MSCs) demonstrate the potential to provide bespoke therapeutics. However, this potential is not without challenges. One significant challenge is maintaining adherence to the substrate in an optimal pluripotent state without premature differentiation. Another challenge is scaling up to higher cell quantities to meet minimum cell therapy requirements while maintaining consistent cell characteristics from scale-down to scale-up.

We present a family of novel bioreactors designed for the expansion and passaging of adherent cells. Adherent cells grow in monolayers on typically planar substrates, requiring conducive microenvironment conditions to prevent poor attachment. This bioreactor allows for different types of coating to be injected as needed based on the adherent cell type. It also leverages microfluidic principles, where fluid/media delivery is controlled such that fluid streamlines generate minimal shear stress. This results in precise microenvironment conditions with low shear stresses, preventing premature detachment or differentiation. Control of shear stresses is

important in adherent culture to maintain expansion efficiency while preserving homogeneous morphology and genetic stability.

Surface area is prime real-estate for adherent cells. Despite the bioreactor being planar, we demonstrate scalability of adherent cell culture from 12cm^2 to 24cm^2 to 48cm^2, whilst maintaining bioreactor geometry. Keeping the bioreactor form factor consistent is crucial for performing scale-down and scale-up culture operations, as this ensures that the local microenvironments near the cells generated by the fluid streamlines remain the same in both smaller and larger bioreactors. Due to the specific design of the bioreactor, we are able to limit shear stresses during feeding regimes, which helps maintain proper adherent stem cell morphologies. As a result, the cells experience the same conditions regardless of the surface area. The bioreactor design also permits automation potential via multiplexing, i.e., fluidically connecting the bioreactors together, allowing for flexible scale-up expansion strategies while reducing labor intensity associated with regular media exchange.

We demonstrate the efficiency and performance of this bioreactor with mesenchymal stem cells and induced pluripotent stem cells. We show that these family of microfluidics-based bioreactors supports enhanced expansion of iPSCs and MSCs compared to conventional vessels with consistent viability, phenotype expression and proliferation characteristics. For MSCs, we obtain comparable cell/cm^2 output at a smaller overall surface area compared to the conventional vessel. However, for iPSCs, we obtain 3-4x expansion vs. conventional vessel whilst maintaining pluripotency. This study presents a novel approach to improving feasibility of scaling-up adherent cell growth to manufacturing levels.

P0852

Characterization and quality controls to support pre-clinical development of innovative lentiviral gene therapies

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Being able to rapidly bring new candidate cell and gene therapies towards the clinical trial stage contributes to biomedical innovation and requires stringent development processes. In this context, it is important to develop analytics early in the process. Characterization and quality control tests provide key information on dosage, manufacturing processes and ultimately define the expected clinical results. It is therefore critical to ensure the quality and reliability of these analytical activities for specific product platforms. The use of HIV-1 derived lentiviral vectors (LV) for gene therapy has demonstrated their potential to address various unmet needs. Here, we describe the analytical methods developed and implemented at ART-TG, an academic laboratory supporting innovation in cell and gene therapy. These methods were developed to ensure the characterization of LV vectors and of LV-genetically modified cells, with the objective of an ISO 9001-certified Quality Management System that will include analytical activities.

The ART-TG laboratory routinely implements robust standardized procedures for the determination of physical and infectious titers of LV productions. LV infectious titers are measured by a digital droplet PCR (ddPCR) technique, shown to be more reproducible and precise than qPCR, using specific primers targeting the integrated provirus to exclude the false contribution of plasmids. Physical titers are measured by p24 ELISA or by a well correlated

nanoparticle tracking analysis. To quantify the residual process-related contaminants such as plasmids in batches of LV or in transduced cells, we developed a ddPCR technique with a limit of quantification (LOQ) of 0.5 copies/µL. A standardized procedure is used to quantify the mean vector copy number per cell (VCN) in human transduced cells using ddPCR and has a LOQ of 0.002 with a CV < 5%, as determined with lentiviral standards consisting of human transduced cells with known VCN. A panel of cell line clones integrating 1, 2, 3 or 5 copies of LV at defined insertion sites, serves as lentiviral standards.

To analyze the vector genomic integration site (IS) in transduced cells, we optimized the LM-PCR library preparation and adapted a bioinformatic pipeline, VISPA2.02, to analyze the Illumina sequencing data. The automated report includes IS mapping, calculation of clonal abundance and indices of diversity, providing annotations pertinent to the transduced cell type e.g. T cells or Hematopoietic Stem Cells. Moreover, microbiology safety tests including bioburden, detection of mycoplasma and endotoxin quantification support the overall product characterization.

Therefore, ART-TG proposes a wide range of analytical LV testings for pre-clinical LV productions and for genetically-modified cells, that is adapted to early-stage products particularly those emerging from academia. In the perspective of offering a more complete IND-enabling package, ART-TG is developing *in vivo* models (humanized mice) to further extend innovative product characterization.

P0853

Scalable and efficient production of Tetracycline-enabled self-repression adenoviral (TESSA®) vectors to support AAV gene therapy manufacture

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1: OXGENE

AAV gene therapy holds great therapeutic promise for a variety of genetic disorders, however, challenges persist in achieving efficient and scalable production to meet the demands of larger patient populations. To address this challenge, we have developed a novel self-silencing helper adenoviral vector system, named TESSA®. This system is designed to deliver all the necessary components for the efficient and contaminant-free manufacture of AAV in serum-free suspension HEK293 cells.

TESSA® is E1/E13-deleted adenoviral vector wherein the Major Late Promoter (MLP) was modified *in situ* to enable self-repression of promoter activity. Specifically, TetR binding sites are inserted into the MLP, while the TetR gene itself is encoded within the late region. This creates a negative feedback loop, where adenoviral structural proteins can only be produced in the presence of doxycycline. This inhibition of MLP activity effectively prevents the expression of adenoviral structural genes during AAV production, thereby preventing the production of adenoviral particles and reducing the quantity and probability of process-related contaminants in the AAV final product.

TESSA® is a versatile and efficient platform for producing AAV vectors, as it offers flexibility through two distinct methods. Researchers can use two TESSA® vectors (TESSA® Duo) to deliver AAV rep/cap genes and the AAV-GOI genome, or they can opt for co-infection with existing AAV-GOI vectors (TESSA® Pro) to facilitate AAV amplification. This flexibility allows for the rapid

and scalable production of cost-effective AAV vectors, achieving up to 20-fold higher yields at >1E+12 vector genomes (GC)/mL and per-cell productivities of >1E+6 GC/cell.

To facilitate the production of the 'critical reagent' TESSA® vectors for AAV manufacturing, we show here the evaluation of a GMP-banked clonal suspension HEK293 cell line and process development optimisation, QC analytics, and stability testing. TESSA® vectors are efficiently generated via single-step assembly cloning method and are routinely batch-produced in 10 L stirred-tank bioreactors, and purified by ion-exchange chromatography, generating sufficient critical reagents to support >50x 200L AAV manufacturing runs. As part of the release testing panel, TESSA® vectors are assessed for microbial safety, vector genome titre, particle potency, genome stability and integrity, and particle stability via dynamic light scattering.

To guarantee the production of clinical AAV gene therapy drugs and expedite product approval, the TESSA® technology is seamlessly integrated with intricate in-house testing capabilities. TESSA® represents a significant advancement in overcoming the challenges associated with AAV vector manufacturing, offering a scalable, efficient, and contaminant-free solution.

P0854

CAR T cells expanded with low interleukin (IL)-2 concentration or IL-7/15 cytokines show subtle differences in T-cell memory subtype composition but demonstrate comparable cytolytic activity *in vitro*

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The culture conditions used in chimeric antigen receptor (CAR) T-cell production have profound effects on the quality of the cells in the final product. We investigated how the strength of the anti-CD3/CD28-microbead-mediated activation signal and cytokine supplementation impact the composition and functionality of cells in CAR-T-cell preparations. Peripheral blood mononuclear cells were isolated from six non-mobilized leukapheresis products from healthy donors by density gradient centrifugation. T cells were enriched and activated with anti-CD3/CD28-microbeads using three bead-to-cell ratios (1:1, 2:1, and 3:1). Subsequently, T cells were transduced with a lentiviral vector carrying a CD19-targeted CAR-gene (CD19-scFv/CD28/z CARs) and expanded for a total of ten days in culture media supplemented with 20 IU/mL interleukin (IL)-2 or 10 ng/mL IL-7 and IL-15. No differences were observed in T-cell expansion or transduction rates. All six methods yielded 100% CD3+ cells with consistent proportions of CD4+ and CD8+ cells in the final products. Naïve T cells (CD95-) and stem cell memory T cells (T_{SCM}: CD95+CD45RO+CD45RA+CD27+) were not detected in any of the CAR T-cell preparations. However, all six methods yielded considerable proportions (mean 59–63%) of T_{SCM}-like cells (CD95+CD45RO+CD45RA+CD27+) in the final products. Interestingly, the proportions of central memory T cells (T_{CM}: CD95+CD45RO+CD45RA-CD27+) were smaller in the IL-7/15 cultures as compared to the IL-2 cultures at all three T-cell activation conditions, mean percentages of T_{CM} cells ranging between 3–4% in the IL-7/15 cultures and 13–15% in the IL-2 cultures. The proportion of effector T cells (T_{Eff}: CD95+CD45RO+CD45RA+CD27-), in turn, was slightly increased in the IL-7/15 cultures as compared to the IL-2 cultures when a bead-to-cell ratio of 3:1 was used for T-cell activation. Despite the subtle differences in T-cell memory phenotype

composition, the IL-7/15 and IL-2 -supplemented cultures contained similar proportions of CD3+ cells expressing T-cell exhaustion and activation markers and demonstrated comparable levels of cytotoxicity against luciferase-expressing CD19+ cells *in vitro*. To summarize, the expansion methods based on a low IL-2 concentration or the combination of IL-7 and IL-15 cytokines yielded CAR T cells with slightly different phenotypic characteristics. However, both conditions generated considerable proportions of cells with early memory phenotypes (T_{SCM}-like and T_{CM}) associated with high therapeutic potential. These findings provide insights into the development of efficient methods for *ex vivo* CAR T-cell production.

P0855

Promoter Screening to Enhance Scalable Recombinant AAV Manufacture from TESSA® Technology

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1: OXGENE

Efficient and scalable production of adeno-associated virus (AAV) vectors is essential for advancing gene therapy applications. TESSA® technology provides an innovative solution through a self-repressing helper adenoviral vector system. This system is uniquely engineered to inhibit the expression of its late adenoviral structural proteins while ensuring the efficient, contaminant-free production of AAV in serum-free suspension HEK293 cells.

The technology is based on E1/E3-deleted adenovirus type 5 vectors, incorporating a Tet repressor binding site into the Major Late Promoter (MLP). This creates a negative feedback loop, where adenoviral structural proteins are only produced in the presence of doxycycline, effectively controlling adenoviral production.

TESSA® serves as a robust and versatile platform for producing AAV, offering flexibility through two distinct approaches. The process variation between the two models differs by the approach in which the gene of interest (GOI) is introduced into the HEK293 cells.

TESSA® vectors are designed to stably express the AAV rep and cap genes and deliver the AAV genome of interest (GOI). TESSA® serves as a versatile platform for producing AAV, offering flexibility through two distinct approaches. A TESSA® vector encoding Rep and Cap can be combined with either another TESSA® vector (TESSA® Duo model) or an AAV (TESSA® Pro model) to deliver the AAV GOI transfer genome for AAV production. This flexibility enables the production of cost-effective rAAV vectors using TESSA® in a rapid and scalable manner, yielding up to 20-fold more AAV vectors with productivities exceeding >1E+6 vector genome copies (GC) per cell.

This study aims to enhance the TESSA® technology further by screening various promoters to boost the expression of AAV Capsid production. Various promoters of varying transcriptional strengths were evaluated to determine their impact on Cap gene expression and AAV productivity. Our goal was to identify the most effective promoter to maximize AAV9 production, thereby further improving the productivity of the TESSA® platform.

A range of TESSA® RepCap9 vectors were successfully generated and evaluated for productivity, propensity for downstream purification, and vector stability. Assessment of AAV9 production using the TESSA® Pro model via co-infection of AAV GOI seed stock with TESSA® RepCap

vectors yielded >3E+12 vector genomes /mL of cell culture. This represented an approximately 5-fold increase compared to first-generation TESSA® RepCap vectors, and >30-fold increase compared to the conventional triple-transfection method. These findings highlight the potential for promoter optimization to substantially improve AAV production yields using TESSA® technology for alleviating the bottleneck in AAV gene therapy manufacture.

P0856

Scalable purification workflow of plasmid DNA using a novel nanofiber adsorbent

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1: Astrea Bioseparations

Plasmid DNA (pDNA) is a critical raw material for many gene therapy treatments and the trend for increasing therapeutic complexity has resulted in the demand for ever larger pDNA sequences. Larger pDNA therapeutics become challenging to process however due to 1) the innate high feed viscosity of these lysates, which cause back pressure during chromatographic purification, and 2) due to the large sequences themselves which require equally large flow paths to successfully be processed. We have developed a next generation chromatography matrix, comprised of a composite electrospun nanofiber with a wide flowpath, that mitigates these problems commonly observed with purification of larger DNA sequences at scale. Commercial processes at scale typically employ two step chromatography processes using IEX and hydrophobic interactions. We have developed a flexible process using a hydrophobic nanofiber adsorbent that can be combined with various IEX prepacked columns either as a capture step OR a polish step to adapt to the varying and growing process needs. Scaling of resin columns is well understood in downstream processing, so in this study we present a single scale DEAE resin capture step followed by a nanofiber adsorbent polish step comparing this novel technology at two scales demonstrating scalable performance, without issues of increased back pressure. We also demonstrate the performance of the nanofiber adsorbent as the primary capture step, allowing the higher capacity and flow speed to easily manage large volume feeds. Our results indicate the potential of the nanofiber adsorbent to deliver a pDNA process that delivers purified plasmid from the milligram to the gram scale.

P0857

Synthetic, enzymatically produced DNA for gene therapy and vaccine applications

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1: 4basebio

The manufacture of high-quality, GMP grade DNA is a major bottleneck in the production of mRNA for use in gene therapy and vaccines. In addition to worldwide lack of capacity and long lead times, complex sequences such as long homopolymeric sequences including long polyA tails

are difficult to propagate in bacteria.

4basebio has developed a proprietary, scalable, fully enzymatic synthesis process for the production of linear DNA constructs via our Trueprime™ amplification technology. The process yields DNA at 1g/L, several orders of magnitude higher than plasmid fermentation yields, allowing a small footprint using benchtop equipment.

The process is size and sequence independent, allowing for large scale production of linear DNA with high yield and purity in less than a week. Unlike plasmid DNA, 4basebio DNA eliminates contamination from endotoxins or host proteins, and excludes bacterial sequences such as antibiotic resistance genes. Complex sequences including ITRs and homopolymeric sequences are easily produced without risk of deletion or recombination.

Currently, we make 4 types of DNA, each with unique application-specific benefits. For mRNA production, opDNA™ can be used directly in IVT processes, achieving significantly higher yields as compared to linearised plasmid, with equivalent capping efficiency and dsRNA impurities. Proinflammatory cytokine/chemokine levels in isolated primary, human PBMCs are comparable to mRNA produced from linearised plasmid.

hpDNA™ can be used in the production of AAV, replacing conventional plasmid tripletransfection. Using hpDNA™, we were able to achieve equivalency in viral genome titres, Full:Empty ratios and infectivity as compared to a plasmid controls, across a range of serotypes.

Finally, proprietary non-viral delivery system, Hermes™, is a nanoparticle vector that can encapsulate a range of payloads, and can be customised to target cells or tissues of interest for a range of applications. The particles additionally offer long term stability at 4 degrees C, overcoming cold chain requirements.

P0858

A comprehensive approach for Comparability of cell and gene therapy product

L Giersch [1] L Dejoint [1]
1: INITS

Comparability of cell and gene therapy products refers to the process of demonstrating that changes in the manufacturing process, formulation, or production site do not adversely impact the safety, efficacy, or quality of the final product. Given the complexity and variability inherent in those biological products, establishing comparability is particularly challenging but essential to ensure consistent therapeutic outcomes. It has also become a full part of the product development life cycle. Indeed, changes into the manufacturing process between the clinical phase 1-2 and phase 2-3 or beyond are almost ineluctable.

As this subject is maturing, several guidance have popped-up in the past few years with the most recent example being the FDA draft guidance on “Manufacturing Changes and Comparability for Human Cellular and Gene Therapy Products” in July 2023. Regulatory agencies are thus looking for the product comparability with more scrutiny and with clearer expectation.

To address those requirements, Inits has implemented a comprehensive approach to analytical product comparability.

The comparability is a multidisciplinary exercise where process and analytical changes are interconnected and should both be evaluated. The first step of comparability demonstration is through an analytical comparability of the product. However, it might not be enough to reach a conclusion regarding comparability which could lead to the need to perform non-clinical and/or clinical studies.

Focusing on the analytical comparability, it is performed in three phases: a) establishment of the protocol, b) carry out of the study and c) data analysis and conclusion formalization through the report.

Some pre-requisites to draft the comparability protocol are needed, such as defining the quality target profile and the critical quality attributes. Material from pre-change process should also be secured to be included in the study.

Inits has developed a comparability protocol defined in 5 steps: a) Process change identification and evaluation, b) Selection of the analytical methods for quality attributes to be used for comparability study, c) Selection of the batches to be used for the comparability (pre-change and post-change), d) Determination of the acceptance criteria for comparability assessment and e) Stability program supportive of the comparability study. This is critical to engage the regulatory agencies early in the comparability study and present them the comparability plan. Inits protocol is a tool helping to prepare this interaction with agency while taking into account their requirements.

The most important work to prepare the protocol is to perform the risk assessment helping to define which change may potentially impact a CQAs, to select the analytical methods that will help to assess the product comparability and perform some head-to-head testing and select an appropriate acceptance criterion using the appropriate statistical tools available. Indeed, the statistical analysis of the batches will drive the conclusion on comparability.

Overall, the comparability of cell and gene therapy product is a complex but essential during product life-cycle and Inits wishes to present a complete overview of its integrated approach towards the comparability assessment of cell and gene therapy product to the pears at the ESGCT 2024.

P0859

Simplified process development: case studies using IsoTag™ reagents for viral vector purification

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1: Isolere Bio Inc.

Process development in viral vector manufacturing is difficult due to quantity and complexity of involved variables. This is amplified by the requirement for scalable production methods that maintain consistency. Additionally, each new vector requires separate process development as specific considerations are required to accommodate size, stability, and ultimate use of each viral vector. In response, we have developed recombinant protein-based reagents that feature engineered affinity tags which simultaneously capture and isolate the target biologics away from contaminants in that solution. These reagents are used in two affinity liquid phase separation (ALPS) processes to optimize variables with minimal volume requirements and then easily scale

those processes using tangential flow filtration. Furthermore, the process relies on only the simple diffusion of a small protein reagent and thus has exceptionally high binding capacity limits for even the largest biologic targets. Tested applications for this powerful core technology include monoclonal antibodies, AAV, adenovirus, lentivirus (LV), and RNA.

Here, we discuss the use of the two complementary ALPS processes for rapid process development: the centrifugation format for small scale purification and high throughput screening (ALPS-CF) and the tangential flow filtration (TFF) format for large scale purification (ALPS-TFF). The IsoTag™ AAV reagent supports small-scale processing through low-speed centrifugation (0.1mL-1L in under 2 hours) with high capture rates (>95%) and recovery rates up to 90%, while larger-scale purification (> 1L) can be achieved using TFF with similar recoveries. The IsoTag™ LV reagent uses a two-step TFF process to streamline downstream purification of lentiviral (LV) material, eliminating any need for chromatography. With over 50% functional recovery, it also achieves a multi-log reduction in host cell proteins and DNA. This process is validated across various feed streams from 1mL-1L, presenting a revolutionary leap forward in LV downstream purification for efficient manufacturing and accelerated development.

Under the lens of two case studies to optimize elution conditions, we used ALPS-CF and ALPS-TFF together to optimize purification processes for AAV and LV at volumes ranging from 100 µL-20 L. In the first, the elution component of the LV purification process was optimized using ALPS-CF to screen buffer options in a DoE, trialing the top candidates in a 50mL ALPS-TFF format, and ultimately implementing the best performing option at the 200mL and 1L ALPS-TFF scales. The process consumed less than 1L of LV harvest while screening 20 buffers, achieving an improved LV recovery across the elution step to > 90%, and reduction in residual IsoTag™ LV from a 1- to a 2-log removal. In the second case study, IsoTag™ AAV was used via a 96-well plate ALPS-CF to screen for compatible elution buffers, with the selected buffer taken to ALPS-TFF to achieve a 20% increase in AAV recovery across the elution step. The selection process consumed < 20mL of AAV harvest and was performed in under one hour.

Taken together, the innovative IsoTag™ reagent platform enables rapid downstream process development for the purification of sensitive biologic substances that can deliver high yields, low contaminants in a fast, easy to implement process.

P0860

The impact of the polysobarte 80 surfactant as a replacement for Triton X-100 in the manufacturing of recombinant adeno-associated virus (rAAV) vectors

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1: Yposkesi - SK Pharmteco

To guarantee the viral safety of biotechnological products derived from cell lines of human or animal origin, one of the techniques for rAAV production involves detergent treatment during cell lysis phase. Triton X-100 has been widely used for this process, but due to its degradation to 4-tert-octylphenols, which have harmful endocrine effects, the use of Triton X-100 is no longer permitted by the European Union (REACH regulations). Because polysorbate 80 is also a stable and non-ionic surfactant but non-toxic and commonly used in domestic and pharmacological applications, this reagent was assessed for its cell lysis and adventitious viral inactivation

properties in the rAAV production. The results have shown that polysorbate 80's efficiency differs from Triton X-100, across serotypes (2, 6, 8 and 9). Although the cell lysis time required (10, 30, 60, 90 and 150 minutes) varies, a 150-minute incubation achieves equivalent viral genome or capsid titers in the rAAV harvest, as Triton X-100. As described in literature, we have confirmed that Polysorbate 80 requires an additional solvent to reach a viral inactivation similar to Triton X-100. Thus, we have tested various concentrations of the tri(n-butyl) phosphate (TnBP) solvent, from 0.025% to 0.1% and determine the minimal dose to obtain a sufficient viral inactivation without affecting rAAV production. Regarding our results, we have confirmed that Polysorbate 80 is sufficient to lyse the rAAV packaging cells, but not to inactivate enveloped viral particles. Therefore, to ensure the viral inactivation of the rAAV harvest, it would be possible to combine a solvent like TnBP to the use of polysorbate 80, which is already implemented for viral inactivation into the manufacturing of drugs that originate from human plasma.

P0861

Transient vs. stable cell lines for AAV manufacturing: a cost modeling approach

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1: Cytiva

To address the growing demand for industrial-scale adeno-associated vector (AAV) production, we recently launched an innovative solution for stable, helper virus-free AAV production. The ELEVECTA producer cell line harbors all relevant components for AAV production stably integrated in its genome, enabling high-titer AAV production upon induction with the inducing agent.

Cell line engineering and optimization is a key area of process optimization in order to increase overall productivities—and to ultimately decrease manufacturing costs of these AAV-based viral vector therapies. Cost modeling can be a useful tool to understand how potential improvements would affect cost of manufacturing and therefore cost per dose. Using the BioSolve software from Biopharm Services Limited, a cost model for AAV manufacturing with the current ELEVECTA producer cell line has been established.

In this work, we have modeled different scenarios of process optimization based on the ELEVECTA producer cell line and compared with our transient cell line process. We have analyzed how they compare in terms of the cost of manufacturing. Scenarios included both fixed-batch throughputs and different yearly throughputs of viral genomes based on modeled diseases.

ELEVECTA is a trademark of Cytiva. BioSolve is a trademark of Biopharm Services Limited.

P0862

Adherent strikes back: Redefining scalability and cost in cell-based manufacturing

T Robert [1]

1: Univercells Technologies by Donaldson

Adherent cell-based manufacturing platforms have long represented the gold standard for cell and gene therapy applications, due to rapid implementation possibilities, straightforward process optimization and high productivities. Nevertheless, developers seeking to achieve cost-effective commercial manufacturing at scale have increasingly sidelined adherent systems as a viable option. This can be explained by their inherent challenges, which include:

- Difficulties associated with precisely measuring biomass in adherent bioreactors;
- Inability to efficiently harvest cells as a product or for seed train generation;
- Historical use of serum containing media introducing regulatory hurdles;
- Poor scalability of legacy systems, leading to low productivities and high costs.

In response to these challenges, manufacturers have shifted to suspension-based platforms. While addressing the above limitations, this approach however faces additional hurdles, such as:

- The need to use suspension-adapted cell lines or microcarrier systems
- Large working volumes complicating key operations and downstream processing
- Non-linear scalability forcing design space compromise and leading to poor productivities
- Difficulties with implementing continuous processing such as perfusion processes

Given these constraints, the field now witnesses renewed interest in innovative platform technologies for large-scale biomanufacturing leveraging the benefits of adherent-based systems while resolving associated issues. This presentation will unveil a novel approach for truly scalable, cost-efficient manufacturing of adherent cells and cell-based products while addressing key challenges associated with biomass estimation, cell harvest and serum-free operations. More specifically, the speaker will:

- Present compelling data on an innovative adherent fixed-bed bioreactor platform supporting linear scalability from 0.5 to 600 m^2 while enabling low-footprint, low-cost biomanufacturing;
- Showcase the use of a novel advanced bioprocess modeling software for accurate cell density estimation and predictive in silico modeling applications;
- Detail extensive datasets demonstrating efficient automated harvest of cells for cell therapy and seed train applications;
- Illustrate how the adherent fixed-bed bioreactor supports serum-free applications for cell growth and production.

Taken together, the data presented supports adherent cell-based manufacturing as a viable, cost-effective option for manufacturing at scale. This new paradigm is expected to facilitate clinical and commercial transition of acutely needed advanced therapies for underserved patients.

P0863

Administration devices management for Gene therapy products a real challenge: A smart approach for in-use stability study and using an integrated risk-based approach as guarantee of quality and safety

L Giersch [1] L Dejoint [1] A Diederichs [1]
1: Inits

Administration devices play critical roles in the development, delivery, and administration of cell and gene therapy products. Control of administration devices for biological products using a risk-based approach ensures quality and safety, which is critical given the complexity and sensitivity of these therapies.

Preparation and administration of biological products is performed through various route of administration and using various devices. Most of the time, several clinical sites are open to conduct the clinical trials and those sites may be used to using various references of devices which may complicate the process of control regarding the administration protocol for the sponsor. Additionally, there is no harmonized guidelines for the control of ancillaries in gene therapy products. The risks related to those devices are related to product adsorption or degradation that would lead to patient risk and to the clinical trial outcome. Thus, Inits has developed a comprehensive process to control, record and justify the use of each reference in a clinical trial.

This process is based on four steps: a) definition of the administration protocol and selection of the administration devices, b) design of the in-use study, c) carry out of the in-use stability study and report of the results and d) implementation of a risk-based approach to approve the clinical site references. A risk-based approach involves identifying, evaluating, and mitigating potential risks associated with the use of devices. This approach will help the industry to improve and simplify the management of administration devices.

The design of the in-use stability is critical to ensure the product compatibility with the administration devices. A smart approach is used to define the study using worst-case parameters (such as preparation duration, holding time, injection time, surface/volume ratio, length…) to ensure unforeseen events during the product preparation and injection are covered and do not jeopardize the reception of the treatment by patients or the treatment safety.

After the completion of the in-use stability study, some clinical sites might require the use of their own material devices that may not have been tested in the in-use study. The decision to use the new device's reference should be justified through a risk assessment and linked with its potential impact on patient safety and product efficacy. This process is firstly defined through a protocol, then the risk assessment is performed using a matrix scoring and the conclusion of the risk assessment is recorded in a memorandum.

The characteristics of the device used to perform the risk assessment can be its composition, size, length or diameter, surface/volume ratio, type of connection… Potential impact of each of those parameters are defined as being low, medium or high risk and the final total risk level is calculated and allow to define if the risk to the patient safety or product efficacy of using this material is low or high allowing to accept or reject the device.

Inits would like the opportunity to present its work on the biological injection device to pears during the ESGCT congress.

P0865

dbDNA™ – Advancing the genetic medicines revolution with synthetic DNA

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1: Touchlight Ltd

Demand for DNA has risen dramatically in recent years, driven by significant growth in the clinical development of nucleic acid therapies. The supply of plasmid DNA has become a major bottleneck in the ever-expanding genetic medicine sector and so the establishment of scalable, faster DNA production technologies are vital for continued growth of these life-changing therapies. Touchlight's enzymatic DNA amplification process offers the potential to eliminate significant capital cost and reduce the footprint of DNA manufacture.

doggybone DNA (dbDNA™) is an enzymatically amplified DNA vector with demonstrated utility in the production of viral vectors, mRNA and cell therapy, and as a DNA vaccine. Touchlight has developed a significant portfolio of IP and developed large-scale DNA manufacturing based on rolling circle amplification (RCA), allowing for the generation of large quantities of high-quality GMP DNA. The ability of this process to generate highly purified, synthetic dbDNA (free of bacterial backbone sequences), offers advantages over conventional and alternative cell free DNA production methods.

Given current g/L yield improvements, reduced footprint of manufacture, low starting template material requirements, and short timescales of production compared with other DNA production methods, the company is uniquely positioned to enable the scale-up of advanced therapies and DNA therapeutics to industrial quantities. This improved manufacturing method addresses a well-understood bottleneck in DNA production capacity.

P0866

A Novel, powerful synthetic DNA for cell and gene therapy applications

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1: Anjarium Biosciences AG

In the last decade, the field of cell and gene therapy has undergone a remarkable expansion. Central to this transformative development is the increased recognition of DNA as an essential input material. As demand for advanced therapies continues to rise, there is increasing need for efficient, timely, and scalable platforms for manufacturing DNA constructs that are used either directly as therapeutic agents or as input material for therapeutic products.

We have developed a novel synthetic, linear, double-stranded DNA, flanked by protective hairpin-ended structures, suited for cell and gene therapy applications. Our DNA provides multiple benefits compared to other DNA constructs options, such as plasmid. Our proprietary one-pot, enzymatic and cell-free production process has a small footprint, seamless scalability and allows production of synthetic DNA constructs of variable size and sequence complexity at high

purity, with faster production times. The final product is devoid of any bacterial sequences. Additionally, as our hairpin-ended structures are not constrained by sequence, they can be customized with either completely novel structure and sequence or inspired in the terminal repeat strategies used in diverse viruses to optimize the functionality of our synthetic DNA.

Here we show our synthetic DNA as a superior alternative for broad cell and gene therapy applications.

- For mRNA production, our off-the-shelf synthetic DNA encoding eGFP with a stable stretch of 140 nucleotides of polyA outperformed its respective plasmid control by leading up to 5-fold higher mRNA yields. The produced mRNA had a superior potency than commercial mRNA from leaders in the field.
- For recombinant adeno-associated virus (rAAV) production, our synthetic DNA resulted in consistently higher AAV titers that its respective plasmid control across a range of serotypes, higher percent of fully loaded viral particles, and higher infectivity, without the risk of reverse packaging.
- For lentivirus vector (LVV) production, our synthetic DNA allowed to produce LVV, encoding the reporter transgene fLuc-t2a-eGFP or the therapeutic gene CD19, with high infectious titer.

Due to its simple and versatile production process and its proven functional performance, our synthetic DNA is well-positioned to replace plasmid DNA as key starting material for cell and gene therapy modalities.

P0867

AAV upstream process optimization in 6- to 96-well format

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Many aspects of recombinant AAV upstream processes can be optimized with resource-friendly small-scale productions. However, testing for the optimal combination of these productivity levers can still be cumbersome and error prone. We have thus developed three tiers of downscaled screening assays to overcome the bottleneck. In the tier one system, we scaled AAV production to a 6-well plate format and used batch affinity purification to harvest AAV for further analysis. While successful, parallelizing the batch purification still proved challenging at scale and some capsids are not compatible with the affinity material. Our tier two system thus omitted the purification step. Instead, we used a salt-active nuclease to remove unpackaged AAV DNA. Determination of AAV titer and genomic purity produced identical results to the tier one approach. Still, throughput was limited. Thus, we developed a tier three approach strictly based on a 96-well plate format. In the third-tier system, AAV encoding secreted luciferase are employed. Producer cells are lysed in-plate and the lysate is used to transduce a permissive test cell line. The AAV titer is then determined as the luciferase activity in the culture media, which, we show, has a linear response to the genomic titer over a broad range. In conclusion, the three developed screening systems provide a useful tool set to engineer AAV production systems, each with specific advantages to drive AAV productivity for providing the next gene therapeutics.

P0868

Next generation Cell&Gene therapy Manufacturing

S Guidi [1]
1: ProPharna

In recent years, new revolutionary ATMP therapies have reached the market, but the technology with which they are produced is poorly automated, dependent on a single supplier and based on open or semi-closed systems, which results in high costs and risks of product contamination as well as failure to reach patient therapy.

Only by reducing production failures and costs will it be possible to make ATMPs more accessible to patients who need these life-saving therapies.

This talk will compare the costs, pros and cons of different manufacturing methods for Cell and Gene Therapy products using different technologies. Thanks to advances in automation, AI integration and efficient containment methods, we may have reached the tipping point.

P0869

Growth Direct® System, an Essential Tool for Environmental Monitoring for the GMP facility and ATMPs Release at CELL*for*CURE by Seqens

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A Russo [1] G Caplat [1] S Ohresser [2] PN Lirsac [1] MO Mignon [1] W Ayassamy-Budoo [1]
1: CELLforCURE by Seqens 2: Rapid Micro Biosystems

CELL*for*CURE by Seqens is currently one of the very few companies worldwide experienced with manufacturing cell and gene therapy drugs that have been administered to large numbers of patients in real life commercial setting. With more than 10 000 m^2 of building surface and 6 independent GMP lines, located close to Paris, **CELL*for*CURE** aims at offering, in a “One Stop Shop” facility, advanced technologies and capabilities in cell therapy development and manufacturing across a large range of cell types.

As with all cGMP facilities, environmental monitoring is a critical parameter for the qualification status of the classified manufacturing area and a key element for the release of safe products.

The Growth Direct® System (Rapid Micro Biosystems) was implemented to handle the environmental monitoring samples of the GMP Facility (qualification of grade A biosafety cabinets, class B and C GMP cleanrooms) and in process environmental monitoring. The system, based on non-destructive microbial detection technology, allows for fully automated incubation, detection, and enumeration of all the samples. The Growth Direct® System detects the cellular autofluorescence of the growing micro-colonies, exploiting the fact that cells fluoresce in the yellow-green when illuminated with blue light, implicating oxidized flavins as the source of much of the signal in this spectral range. The Growth Direct® System distinguishes growing microbial colonies from fluorescent particles by superimposing images from several consecutive readings and subtracting fluorescent spots that do not increase in size. The technology is non-destructive, as the illumination with the blue light does not damage microorganisms. This important benefit

means that colonies can be subsequently identified by standard microbiological techniques. As the detection system's CCD (Charge-Coupled Device) chip offers better resolution and greater sensitivity than the human eye, with reading the samples every 4 hours, colonies can be detected earlier, in as little as 24 hours. Internal validation study allows a final reading after 56 hours of incubation instead of at least 120 hours (5 days minimum) needed in the classic incubation methodology. Each Growth Direct® System has two Incubators with a sample capacity of 330 plates each. The total incubation capacity is 2112 plates when utilizing all 4 instruments validated in the Quality Control microbiology laboratory. Until the implementation of a rapid microbial method (RMM), the compilation of the environmental monitoring data was a major bottleneck for the release of ATMP product like an autologous CAR-T cell product, when manufacturing timelines are critical. The Growth Direct® System has drastically accelerated the vein-to-vein therapeutic transfer back to the patient reducing overall duration by 20%.

P0871

Enhancing Stability and Delivery of Alpha-Galactosidase A for Fabry Disease Treatment

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1: Amicus Therapeutics

Alpha-galactosidase A (GLA) is a lysosomal enzyme that hydrolyses terminal galactose residues on diverse cellular substrates. Mutations in *GLA* lead to Fabry disease, an X-linked lysosomal storage disorder characterized by multisystem organ involvement, including kidney and heart pathologies**.** Approved treatment options for Fabry patients include enzyme replacement therapy (ERT) using systemically infused recombinant human alpha-Gal A (rhα-Gal A) and an orally administered molecular chaperone for patients with amenable *GLA* mutations. Recently, adeno-associated viral (AAV) gene replacement approaches have emerged as a promising therapeutic approach. However, limitations with both ERT and gene therapies include variable uptake into different disease-relevant tissues and short circulating half-life of GLA enzyme at neutral pH. Here we present a newly developed Amicus STABLE protein engineering platform (Stability Through Advanced Biologic Learning and Engineering) to generate novel GLA variants with enhanced stability. Through iterative rounds of engineering, we created constructs demonstrating superior half-lives, enhanced expression, and higher activity *in vitro.* AAV delivery of our lead engineered GLA variants in non-human primates resulted in durable long-term expression with a favorable safety profile. These data demonstrate potential solutions to overcome current limitations in developing effective therapies for treating Fabry disease, and the Amicus STABLE engineering platform holds promise for extending its application to a broader range of genetic conditions.

P0872

Delivering mitochondrial base editors with lipid nanoparticles and virus-like particles

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CRISPR/Cas9-based DNA editing has greatly accelerated the development of gene correction therapies, but it offers no solution for disorders caused by mitochondrial DNA mutations. DddA-derived cytosine base editor (DdCBE) is a recently developed technology specifically designed for C-to-T editing of mitochondrial DNA (mtDNA) and may thus form the basis for mitochondrial gene correction strategies. Since its development, DdCBE has been used to introduce and correct various pathogenic variants in cell lines, but effective delivery of mitochondrial gene editors with clinical-grade delivery systems is little explored. Our aim was to develop delivery methods for DdCBE that enable efficient gene correction in accurate cell models for mitochondrial disease.

To enable transient but high cellular expression of DdCBE, we designed constructs to *in vitro* produce mRNA that encode DdCBE. Transfection with DdCBE-mRNA compared to plasmids improved transfection efficiency and reduced cytotoxicity while yielding similar mtDNA editing efficiency levels. Lipid nanoparticles charged with DdCBE-mRNA enabled efficient mitochondrial gene editing in patient cells. Delivery with virus-like particles led to clear expression of DdCBE protein in transduced cells, a fraction of which localized in the mitochondria, resulting in moderate yet detectable mitochondrial gene editing.

These results show that transfection with mRNA instead of plasmids greatly reduces cytotoxicity without impeding editing efficiency. Moreover, we demonstrate that lipid nanoparticles are an efficient delivery method for DdCBE to achieve mitochondrial gene correction in patient cells. Delivery with mRNA virus-like particles also resulted in mitochondrial gene editing, which sets the stage for further optimisation of virus-like particle-based delivery. This study demonstrates the feasibility of clinically relevant delivery methods for developing curative therapeutic strategies to the benefit of mitochondrial patients.

P0873

First patient treated in the phase 1–2 trial of AAVLK03hOTC gene therapy for ornithine transcarbamylase deficiency in children

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Unit, Children's Medical Research Institute and The Children's Hospital at Westmead, Wentworthville, New South Wales, Australia

Ornithine transcarbamylase deficiency (OTCD) is a rare X-linked urea cycle disorder that leads to recurrent metabolic decompensations characterised by hyperammonaemia, high glutamine levels, liver dysfunction and progressive encephalopathy leading to long-term neurological damage. Conventional treatments have limited efficacy and whilst liver transplantation is effective, it has associated morbidity and mortality, requires lifelong immunosuppression and is challenged with a shortage of organ donors.

We developed the adeno-associated virus (AAV)-based gene therapy AAVLK03hOTC encoding a human codon-optimised OTC gene and pseudoserotyped with highly human hepatotropic LK03 capsid to target the liver and restore functional expression of OTC enzyme. Preclinical *in vitro* and *in vivo* studies demonstrated restoration of functional OTC activity in OTC deficient hepatocytes following treatment with AAVLK03hOTC.

A 26-week GLP toxicity and biodistribution study in naïve Cynomolgus monkeys demonstrated elevated liver OTC activity 26 weeks after administration of AAVLK03hOTC in the absence of notable toxicological alterations. Whilst AAVLK03hOTC was expressed in almost all fluids and organs analysed, highest levels were in the liver.

“Halting Ornithine transcarbamylase deficiency with Recombinant AAV in ChildrEn” (HORACE) is a phase I/II clinical trial based on a single administration of AAVLK03hOTC to paediatric OTCD patients with severe phenotype which aims to achieve normalisation of liver metabolism, thereby reducing acute risk of morbidity and mortality from hyperammonaemic decompensations.

Three doses (6x10^11vg/kg, 2x10^12vg/kg and 6x10^12vg/kg) are proposed in the dose escalation part of the trial.

Posterior risks of Dose Limiting Toxicity (DLT) calculated after data on the incidence/absence of DLT will be collected. For dose-escalation cohorts, all patients will be 6-16 years to ensure homogeneity between cohorts. Based upon predicate data from other AAV gene therapy trials, there is a risk of asymptomatic transaminitis likely caused by T-cell mediated immune response to AAV, readily controlled by immunosuppression. Following escalation, recruited patients will be adaptively allocated to dose according to model-based simulation. After the initial 12 month-follow up period participants will enter a 5-year follow up study under a separate protocol.

The first patient treated with low dose (6x10^11vg/kg) AAVLK03hOTC gene therapy was a 10-year-old male with late-onset OTCD. Previous history included recurrent episodes of hyperammonaemia treated with dietary protein restriction and daily ammonia scavengers. The patient tolerated the gene therapy administration remarkably well. Two weeks after administration he presented with hyperammonaemia and encephalopathy caused by mycoplasma pneumonia encephalitis unrelated to gene therapy, followed by full neurological recovery. Significant reduction in ammonia and glutamine levels were noted 6 and 8 weeks after gene therapy administration respectively, which allowed initiation of ammonia scavenger withdrawal. 7 months after gene therapy administration the patient developed raised liver transaminases >2 times above upper limit of normal. He remained asymptomatic but treated with prednisolone and tacrolimus which achieved normalisation of the transaminases within 2 weeks. Glutamine and ammonia levels remained below the lower limit of normal and reduction of ammonia scavengers to <75% of pre-treatment levels was achieved whist maintaining metabolic stability and stable dietary protein intake. Further withdrawal of conventional treatment is in progress.

P0874

Repeated dosing of AAV-mediated liver gene therapy in juvenile rat and mouse models of Crigler-Najjar syndrome type I

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Crigler-Najjar syndrome is an ultra-rare monogenic recessive liver disease caused by *UGT1A1* gene mutations. Complete UGT1A1 deficiency results in severe unconjugated hyperbilirubinemia in newborns that, if not treated, may lead to brain damage and death. Treatment is based on intensive phototherapy, but its efficacy decreases with age, rendering liver transplantation the only curative option. AAV-mediated gene therapy has shown long-term correction in adult patients, but loss of viral DNA and therapeutic efficacy are expected in younger patients associated to liver growth. Effective vector re-administration is hindered by anti-AAV neutralizing antibodies generated during the first administration. Here, we investigated AAV-vector re-administration by modulating the immune response with rapamycin-loaded nanoparticles (ImmTOR) in Gunn rats (*Ugt1a*$^{-/-}$) and *Ugt1a*$^{-/-}$ mice. We administered a liver-specific AAV8 vector expressing a codon-optimized h*UGT1A*1 cDNA (1.0E11 vg/kg) in P25-P28 mutant animals and, upon loss of efficacy after 3 to 5 weeks, a higher second dose (1.0E12 or 5.0E12 vg/kg) was given. ImmTOR co-administration reduced anti-AAV NAbs and IgGs generation in male animals of both models allowing effective re-dosing, underscored by a significant and long-term decrease in plasma bilirubin, although efficacy was affected by low-titer residual anti-AAV antibodies suggesting that re-administration in patients may require combination with other methods.

P0875

Adeno-associated virus serotype 9 expressing human propionyl-CoA carboxylase, subunit beta for the treatment of patients with propionic acidemia resulting from a deficiency of PCCB

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Propionic acidemia (PA) is rare autosomal recessive metabolic disorder caused by defects in the mitochondrial localized enzyme propionyl-CoA carboxylase (PCC). The PCC enzyme is composed of six α- and six β-subunits with causative variants occurring at equal frequencies in either the nuclear encoded *PCCA* or *PCCB* gene. Individuals with PA can suffer from poor growth, lethal metabolic decompensations, and cardiomyopathy despite current medical management, which has led to the pursuit of gene therapy as a new treatment option for patients. Here we use

several murine models of PA caused by PCCB deficiency to generate pre-clinical data to support an FDA Investigational New Drug (IND) Application as part of the Accelerating Medicine Parternship (AMP®) Bespoke Gene Therapy Consortium (BGTC) program sponsored by the Foundation for the National Institutes of Health. Systemic delivery via retroorbital injection of AAV9-hPCCB at dose range of $2.5x10^{13}$ to $1x10^{14}$ vg/kg to neonatal lethal, juvenile lethal and hypomorphic PCCB PA mice, representative of the spectrum of genetic mutations and disease phenotypes reported in PA patients, resulted in significantly increased survival, reduced disease related metabolites, improved growth, and protection against metabolic stress in comparison to the respective untreated mutant mice. Based on disease severity, murine models of PA were treated as neonates, juveniles, or adults, which is reflective of the age range of the PA patient population. PA patients experience early mortality, therefore the significant improvement in survival strongly supports the efficacy of AAV9-hPCCB therapy while the reduction of disease related metabolites observed after AAV9-hPCCB treatment provides evidence of increased PCC enzymatic activity and may be clinically beneficial because these metabolites are thought to be toxic. Lastly, the resistance to metabolic stress in a murine model of PA is clinically relevant because patients are known to experience potentially lethal metabolic decompensation in response to stressors. Our pre-clinical data demonstrate the efficacy of AAV9-hPCCB gene therapy as a treatment for PA caused by PCCB deficiency and support the advancement toward clinical translation.

P0876

Preclinical development of a hematopoietic stem cell gene therapy for mucopolysaccharidosis type II

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Mucopolysaccharidosis type II (MPS II), also known as Hunter syndrome, is an X-linked recessive lysosomal storage disease (LSD) caused by a deficiency of iduronate-2-sulfatase (IDS). The progressive accumulation of glycosaminoglycans, which are substrates of IDS, leads to various symptoms such as skeletal deformities and central nervous system (CNS) involvement. Hematopoietic stem cell gene therapy (HSC-GT) is a promising treatment with long-term efficacy for correcting systemic phenotypes in several LSDs. We have previously demonstrated that HSC-GT using lentiviral vectors can improve systemic biochemical alterations, memory function, and skeletal abnormalities in a murine model of MPS II. In this study, we attempted to develop a cell manufacturing process for gene-modified human HSC and performed a preclinical safety study using semi-GMP-grade products. After gene transduction of CD34+ cells derived from PBMC, the cells showed significantly higher IDS activity than untransduced cells. A colony-forming unit assay indicated high transduction efficiency in the manufactured CD34+ cells. No differences were reported between gene-modified HSC-treated and -untreated NOG mice when we outsourced CRO for general toxicology and histopathology studies. In addition, lentiviral vector integration analysis revealed an oligoclonal pattern in the genome of bone marrow cells from NOG mice after HSC-GT, and integration near the MECOM locus, albeit at an extremely low frequency. Our data provide a preclinical safety profile of HSC-GT for MPS II.

P0877

Gene therapy for SENDA/BPAN with AAV vector and detection of biomarkers

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Static encephalopathy of childhood with neurodegeneration in adulthood / β-propeller protein-associated neurodegeneration (SENDA/BPAN) is a neurodegenerative disorder with brain iron accumulation caused by the variants of *WDR45*. Although *WDR45* is one of the core autophagy-related genes, no other variants of core autophagy-related gene show abnormalities of iron metabolism. We elucidated that pathology of SENDA/BPAN is based on the impaiement of ferritinophagy a ferritin-specific autophagy (Brain Commun. 2022).

We conducted gene transfer using adeno-associated virus vectors expressing *WDR45* (AAV-CMV-WDR45). In vitro studies showed that gene transfer recovered ferritinophagy and corrected compensatory response of iron transporters. In the Brain of WDR45 defficient mice, iron accumulation and neuronal loss were detected, similar to the patient's pathology. Gene transfer of *WDR45* deficient mice improved these neuronal pathologies. We are planning a non-clinical studies to verify the efficacy of AAV-CMV-WDR45 using pig models.

In SENDA/BPAN, iron is gradually deposit mainly in basal ganglia and symptoms progress. Therefore, Early diagnosis and treatment are important. We explored biomarkers which are useful for early diagnosis and evaluation of treatment using patient-derived fibroblast and the serum of WDR deficient mice. According to multi-omics analysis, proteome, metabolome, and transcriptome, we identified several candidates with potential clinical applications. In particular, substance X would be the most valuable candidate as it was at high level in *WDR45* deficient mice and at low level in wild type and AAV-CMV-WDR45 treated *WDR45* deficient mice. We will continue validation in mice and confirm whether it can be used as a diagnostic criterion in human specimens. In addition, we aim to establish a measurement system and introduce it into screening.

P0878

Improving gene therapy tools for Pompe disease

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Pompe disease is a rare autosomal recessive disorder caused by mutations in the lysosomal acid alpha-glucosidase enzyme (GAA), responsible for the catabolism of glycogen to glucose. This deficiency causes the accumulation of glycogen in multiple tissues, mainly affecting skeletal and cardiac muscle as well as the central nervous system. Apart from the lack of GAA activity and the

accumulation of glycogen into the lysosomes, this disease is characterized by defective autophagy and reduction of cation-independent mannose 6-phosphate receptors (CIMPR). These receptors are involved in the enzyme uptake into the cells and trafficking towards the lysosomes. Regarding current therapies, the only standard treatment is the enzyme replacement therapy (ERT). However, not all patients respond to ERT, it is expensive, and it is frequent the development of immune responses that prevent its re-administration. This shows the actual necessity of new alternative therapies. In this line, gene therapy has emerged as an alternative treatment for different monogenic diseases such as Pompe disease. Consequently, the global aim of this study is to generate new tools for gene therapy of Pompe disease patients.

For that purpose, improved 3rd generation lentiviral vectors have been designed including GAA or GFP sequences under different promoters and including different leader peptides in order to increase secretion, uptake and lysosomal destination of GAA by target cells. These lentiviral vectors have been employed to genetically modify THP-1 cells, a myeloid cell line that can be differentiated into macrophages when treated with PMA. The potential advantage of this strategy includes that macrophages could migrate to central nervous system, providing therapeutic protein to this affected system. Genetically modified THP1 cells have been studied to select the best construct in terms of GAA overexpression, secretion and uptake. For that, engineered-GFP expression was compared between control and differentiated cells. Additionally, secreted-GFP were measured by ELISA and a Transwell System was employed to study GFP uptake. Furthermore, THP-1 cells were stained with LysoTracker and confocal analyses were performed to assess GFP location in both donor and receptor cells.

To improve the efficiency of our selecting vectors *in vivo*, in parallel, we performed *in vivo* experiments in B6-129 Pompe mice with two objectives: to increase CIMPR expression levels and to alleviate their autophagic pathology. For that purpose, we treated these animals, as described in literature, with salmeterol or L-arginine, both administered in drinking water to mice for 18 or 6 weeks, respectively. As expected, our results showed that salmeterol administration enhances CIMPR expression in quadriceps, heart and diaphragm. Additionally, L-arginine restores p62 levels in heart and quadriceps.

In conclusion, in this study it has been optimized different methodologies with the final aim to propose a combined treatment that could lead to better benefits that those that can be obtained separately. This combination therapy has the potential to emerge as a more efficacious therapeutic strategy of Pompe disease.

P0879

Sub-optimal outcomes from a compassionate access program of ex-vivo gene therapy for neuronopathic gaucher disease - why clinical gene therapy programs should be developed in clinical-academic environments rather than as primary commercial endeavours

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Gaucher disease (GD) is a lysosomal storage disorder resulting from *GBA1* pathogenic variants which cause deficiency of glucocerebrosidase. There are both neuronopathic and non-neuronopathic forms (GD2 and GD3, and GD1 respectively). Treatment is with IV Enzyme Replacement Therapy (ERT) delivered bi-monthly for life. ERT does not correct neuronopathic disease as it doesn't cross the blood brain barrier. Absence of therapies in GD2 and GD3 constitutes an unmet need in this disease.

A lenti-viral, commercially developed, ex-vivo gene therapy program for GD was developed, initially targeting GD1 (as the larger population in developed countries). Given the unmet needs of the GD3 population, a further program using the same product (AVR-RD-02) was under development in 2021-23. Pre-clinical trial engagement enabled us to access compassionate therapy for two siblings with GD3. Here we report their outcomes.

Patient 1 was 11yrs at intervention, he had typical GD3 and had been previously treated with ERT and substrate reduction therapy. He had multiple neurological clinical signs, intellectual impairment, seizures and symptomatic visceral disease. He underwent mobilisation and leukapheresis of HSCs which were then transduced with AVR-RD-02. Conditioning was with myeloablative busulfan. Engraftment was prompt and sustained (neutrophils > 1×10^9/L and platelets > 50×10^9/L, at day 9). He had a single episode of neutropenic fever and there was no transfusion need. The patient remained off all Gaucher-specific therapy following AVR-RD-02 administration. Peripheral and bone marrow Vector Copy Number data confirmed sustained engraftment at day 56 and later at day 582 (>1yr). Peripheral leukocyte enzyme activity reached normal healthy control range at 6 weeks (1.08nmol/mg/hr; healthy control 1.0 – 5.0 nmol/mg/hr); at 13 months was 3.5nmol/mg/hr and 1.87nmol/mg/hr at 2yr 9mo. A parallel fall in disease biomarkers to normal ranges was also achieved for the first time ever and has been sustained (2yr 9mo).

Patient 2 was 3.5yrs at time of intervention and had a lower Gaucher disease burden reflecting his younger age. He received the same product and conditioning as his older sibling. Haematological engraftment was achieved at day 13. He did require some blood product transfusions and had a single episode of febrile neutropenia (culture negative). The company providing compassionate access to therapy withdrew the program in 2023 and so no post dosing VCN data was available, although baseline VCN was low. Peripheral leukocyte enzyme activity did not reach normal healthy control range until 11mo; all Gaucher specific therapy had been stopped following haematological engraftment and consideration was given to restarting ERT at 6month follow up. Disease biomarkers fell in parallel with rise in leukocyte enzyme activity but remain elevated at 1year.

We present this data to highlight the importance of developing therapies with a patient-centred motivation. The vector here was suboptimal and the engineered product had low VCN, gene expression did not compensate for this low VCN (much lower enzyme overexpression, compared to our other GT programs in LSD). The commercial model did not permit optimisation and our patient community is without a GT programme to access, their need remains unmet.

P0880

Atidarsagene autotemcel (autologous hematopoietic stem cell gene therapy) preserves cognition, language, and speech and slows brain demyelination and atrophy in early-onset metachromatic leukodystrophy

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Metachromatic leukodystrophy (MLD), a demyelinating lysosomal storage disorder caused by arylsulfatase A (ARSA) enzyme deficiency, results in progressive neurological impairment and early death. In advanced MLD, patients lose all motor and cognitive skills and communication, severely impacting their quality of life (QoL).

Atidarsagene autotemcel (arsa-cel) consists of autologous $CD34^+$ cells transduced *ex vivo* with a lentiviral vector encoding the human *ARSA* cDNA with constitutive expression driven by a human *PGK* promoter, infused intravenously after busulfan conditioning. We previously presented primary efficacy outcomes from 37 patients with early-onset MLD (18 late infantile [LI, onset ≤30 months], 19 early juvenile [EJ, onset between 30 months and <7 years]) treated with arsa-cel across two prospective clinical trials (NCT01560182, NCT03392987) and an expanded access program. Here we present outcomes of arsa-cel on cognitive function, language, speech, and brain MRI severity scores in pre-symptomatic LI (PSLI), pre-symptomatic EJ (PSEJ) and early symptomatic EJ (ESEJ, able to walk independently and without cognitive decline before treatment) MLD, compared to a natural history (NHx) cohort of 43 untreated early-onset MLD patients (26 LI, 17 EJ).

Median follow-up was 6.76 years (range 0.64-12.19). Most arsa-cel treated patients (81%, 30/37) maintained performance and language standard scores representing normal (≥85) or mildly impaired (≥70 and <85) cognitive and language skills at the last available neuropsychological assessment. In contrast, all NHx patients develop severe cognitive and language impairment early in their disease course. Accordingly, the risk of experiencing confirmed severe cognitive impairment or death was markedly reduced for the arsa-cel PSLI ($p<0.001$), PSEJ ($p=0.044$), and ESEJ ($p=0.003$) treated subgroups versus MLD subtype-matched NHx patients. Furthermore, arsa-cel reduced the risk of experiencing complete loss of speech in PSLI ($p<0.001$), PSEJ ($p=0.042$), and ESEJ ($p=0.032$) treated subgroups versus NHx patients, most of whom lost all speech. Brain MRI total adapted Loes scores in arsa-cel treated patients were markedly lower at 5 years post-treatment compared to age-matched NHx patients [PSLI (n=8), $p<0.001$; PSEJ (n=3), $p<0.001$; ESEJ (n=3), $p=0.025$] and were stable over time, indicating prevention or slowing of brain demyelination and/or atrophy in treated patients. Additionally, several patients (1 PSLI, 2 PSEJ, 1 ESEJ) showed slight decreases in brain MRI scores after treatment that can be attributed to improved brain myelination.

With up to 12 years follow-up, arsa-cel shows benefits to treated patients' QoL by preserving cognition, language abilities, and speech, supported by evidence from brain MRI.

P0881

Perinatal CRISPR/Cas9 mediated targeted gene insertion at the *Ass1* native locus provides effective and durable rescue in a lethal mouse model of citrullinemia

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Citrullinemia type I is a life-threatening urea cycle disorder impacting approximately 1 in 57,000 live births. It is a monogenic condition arising from deficiency of argininosuccinate synthetase (*ASS1*). Left untreated, citrullinemia can lead to serious metabolic decompensation, marked elevation in blood ammonia, and early death. Today, perinatal newborn screening helps to rapidly identify individuals with citrullinemia, enabling prompt intervention in the form of dietary protein restriction and nitrogen scavenger therapy. However, lifelong dietary protein restriction and frequent administration of nitrogen scavenger medication poses significant adherence challenges. Therefore, many patients receiving standard of care continue to suffer from periodic bouts of hyperammonemia and metabolic decompensation that can progressively lead to irreversible neurological, behavioral, and cognitive sequelae. Liver transplantation is often curative but poses its own limitations and complications. The monogenic nature and curative potential of liver transplantation make citrullinemia an ideal indication for liver-directed gene therapy. However, episome-based hepatic AAV gene therapies are often ineffective when administered early in life due to vector dilution. This poses a significant obstacle to episome-based liver-directed gene therapies for citrullinemia where early intervention is critical to prevent irreversible neurological manifestations of the disease. To address known obstacles of episome-based liver gene therapies, we have developed an alternative, CRISPR-based platform for hepatic gene replacement. We aimed to apply this technology to develop a durable and effective liver-directed targeted gene insertion treatment for citrullinemia that can be administered during the perinatal period. Our approach uses lipid nanoparticles to transiently deliver Cas9 mRNA and an *Ass1*-directed guide RNA, co-administered with AAV containing a promoter-less ASS1 partial cDNA for insertion into the native *Ass1*chromosomal gene locus. Using this approach, expression of the inserted therapeutic transgene is controlled at the native locus via endogenous regulatory elements. Proof-of-concept studies performed in the lethal *Ass1*^{fold} citrullinemia mouse model, in both young adult (P28) and newborn (P7) mice demonstrate that *Ass1* native locus gene insertion was highly effective at durably reducing blood ammonia and citrulline. Histological and molecular analyses revealed efficient targeting and correction of the *Ass1* locus in a significant fraction of hepatocytes. Importantly, we observed comparable efficacy not only in young adult (P28) mice, but also in newborn (P7) mice where significant hepatocyte proliferation is taking place. This was in stark contrast to ASS1 episome-based AAV gene therapy. As expected, we observed minimal correction of ammonia and citrulline levels when administering an ASS1-encoding AAV episome to P7 *Ass1*^{fold} neonates, and only transient efficacy in young adult P28 *fold* mice. We also observed important differences in zonal hepatocyte correction between *Ass1* native locus gene insertion and AAV episome treatment approaches. *Ass1* native locus gene insertion led to preferential ASS1 restoration in peri-portal hepatocytes with high urea cycle activity, whereas AAV episome

approach led to ASS1 restoration preferentially in peri-central hepatocytes with low urea cycle activity. Taken together, these results demonstrate that ASS1 gene insertion may provide an effective and durable treatment that can be administered early in life to promptly halt the onset and progression of irreversible manifestations of citrullinemia.

P0882

An innovative platform approach for the parallel development of HSPC-GT for rare/ultra-rare lysosomal storage disorders with severe skeletal manifestations

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Lysosomal storage disorders (LSDs) are caused by the deficiency of a specific lysosomal enzyme, causing the accumulation of undigested macromolecules in multiple tissues. This accumulation results in severe multi-organ damage and inflammation. Currently approved therapies provide an external source of therapeutic enzyme available for uptake by diseased cells, but they show limited accessibility to poorly vascularized or barrier-protected tissues (1-3). Previous clinical data obtained by our Institute proved the safety and efficacy of Hematopoietic Stem and Progenitor Cell-Gene Therapy (HSPC-GT) for LSDs with severe neurological and skeletal involvement (NCT01560182, NCT03488394). These results suggest that gene-modified HSPC-progeny releases supraphysiological levels of the therapeutic enzyme, possibly increasing its penetration into skeletal and brain tissues (4-6). Building on this experience and the common pathological mechanisms of LSDs, we are developing a platform approach of HSPC-GT to treat a group of rare/ultra-rare LSDs with severe skeletal involvement (MPSIVA, MPSIVB, and a-Mannosidosis), utilizing the lentiviral vector (LV) backbone previously employed (4,5) and a platform standardized transduction protocol. Based on these characteristics, we optimized the Chemistry, Manufacturing and Controls activities, Non-clinical and Clinical development plans to generate a complete dataset for the lead disease (MPSIVA), complemented by disease specific data for the other indications with the aim of submitting a single Clinical Trial Application (CTA) to initiate clinical evaluation. This move towards a simultaneous and parallel approach, may enable the development of novel therapeutic interventions for rare and ultra-rare diseases to be more sustainable. To this aim, we tested the LVs encoding for each specific enzyme (LV-GALNS, -enhGLB1, and -MAN2B1) in human HSPCs, showing that the progeny of transduced cells (myeloid cells and osteoclasts, serving as circulating and tissue-resident enzyme source) are capable of releasing supraphysiological amounts of the therapeutic enzyme, which is sufficient to cross-correct patients' cells, including skeletal cells. Using the clinical-grade LV, we selected the platform transduction protocol showing high transduction efficiency (VCN >2 and > 80% positive colonies) and no signs of toxicity in vitro and in vivo. A platform GLP study for parallel evaluation

of enzyme overexpression toxicity is ongoing. In this study, we compare wild-type mice transplanted with murine hematopoietic stem cells transduced in vitro to overexpress each specific enzyme to a single control group transplanted with un-transduced cells. Moreover, disease-specific proof of concept studies started to evaluate the safety and efficacy of HSPC-GT compared to standard HSPC transplantation. Our preliminary data support the platform development of HSPC-GT for LSDs and its clinical application.

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P0883

Pancreas-directed gene therapy for maturity-onset diabetes of the young type 3 (MODY3): Moving from mice to large animals

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Maturity-onset diabetes of the young (MODY) are a group of monogenic diabetes characterized by onset of hyperglycemia at an early adult age, normally before 25 years. MODY3, the most common type of MODY, is caused by mutations in the gene encoding for the transcription factor hepatocyte nuclear factor 1A (HNF1A). MODY3 patients are usually treated with sulfonylureas, but they tend to become unresponsive to these drugs and adverse effects associated to chronic administration of sulfonylureas have been reported. Presently, there are no current treatments addressing the root cause of MODY3. Here, using a unique new strategy based on the CRISPR/Cas9 technology, we generate a novel MODY3 mouse model that recapitulates the main alterations observed in human patients. Local administration of AAV vectors encoding Hnf1α to the pancreas of MODY3 mice via intraductal delivery resulted in Hnf1α expression in β-cells, which upregulated the expression of HNF1A main target genes, such as GLUT2 and L-PK. AAV-Hnf1α-mediated gene therapy counteracted hyperglycemia, increased insulinemia, improved glucose tolerance and enhanced insulin secretion by pancreatic β-cells, thus reverting the MODY3 phenotype. As a first step towards translation, intraductal delivery of AAV vectors to Beagle dogs resulted in efficient transduction of pancreas, including endocrine cells, in the absence of adverse events or toxicities. Overall, these results underscore the potential of the AAV-Hnf1α gene therapy to treat MODY3 and support its clinical translation.

P0884

Profiling AAV integration in gene therapy and genome editing for Wilson disease

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Wilson Disease (WD) is an autosomal recessive genetic disorder caused by mutations in the *ATP7B* gene and characterized by toxic copper accumulation in liver and extrahepatic organs. Liver-directed gene therapy using adeno-associated viral (AAV) vectors offers potential treatment and is currently undergoing clinical trials. AAV genome typically remains episomal, but it could also integrate at low frequency in the host genome. While raising concerns in terms of genotoxicity, AAV integrants may also contribute to therapeutic effect. AAV integration preferentially occurred at DNA double-strand breaks (DSB) and we found that human *ATP7B*$^{-/-}$ hepatocytes were more susceptible to DSB generation in response to copper challenge compared to wild-type cells. Consistently, increased DSBs were observed in ATP7B-deficient human and mouse livers relative to controls. Surprisingly, transcriptional upregulation of genes involved in homology-directed repair (HDR) was found in ATP7B-deficient human hepatocytes and mouse livers.

To investigate the effect of copper-mediated DNA damage on AAV integration, we analyzed the integration profiles of AAV-based classic gene therapy and genome editing in *Atp7b*$^{-/-}$ mice. Genome editing was achieved by nuclease-free targeted integration of a promoterless human mini-ATP7B cDNA into the albumin locus (Alb-ATP7B) through HDR. The gene replacement vector included a human mini-ATP7B cDNA under the control of a liver-specific promoter (HLP-ATP7B). Control vectors bearing GFP (Alb-GFP and TBG-GFP) were also studied. AAV integration sites (IS) were retrieved from liver genomic DNA using Sonication Linker-Mediated (SLiM) PCR and the Recombinant Adeno-Associated Vector Integration (RAAVioli) pipeline.

Livers injected with the HLP-ATP7B vector displayed the highest IS number, a 100-fold increase compared to TBG-GFP controls, suggesting that these integration events provided a selective advantage of corrected hepatocytes. Conversely, similar IS numbers were observed in mice injected with genome editing vectors (Alb-ATP7B and Alb-GFP). Clonal diversity measured by the Shannon diversity index indicated higher polyclonality in HLP-ATP7B injected mice, whereas genome editing vectors showed a more oligoclonal profile. Gene enrichment analysis revealed an over-representation of genes associated with liver damage and dysfunction. The albumin *(Alb)* gene was the most targeted in genome editing experiments and showed high targeting frequencies in HLP-ATP7B injected mice. However, IS distribution within the *Alb* gene varied depending on the vector used, with Alb-ATP7B integrations predominantly aligning with the direction of *Alb* gene transcription, thus potentially contributing to selective advantage. *Ppp1r12b*, *1700048O20Rik* and *Cps1* also emerged as frequently targeted genes by both gene therapy and genome editing vectors. TBG-GFP IS were often found in *Atp7a*, a paralogue of *Atp7b* with low hepatocyte expression, suggesting TBG-GFP integration may transactivate *Atp7a*, compensating for ATP7B function and conferring a selective advantage to hepatocytes. Consistently we found increased hepatic *Atp7a* expression in TBG-GFP-treated mice, compared to mice injected with HLP-ATP7B and to healthy controls

In conclusion copper-mediated DNA damage may increase off-target and random integration events, but it could also enhance on-target integration of genome editing vectors through

upregulation of HDR machinery. These findings underscore the importance of disease conditions in determining AAV integration profiles and highlight the need for further investigation into the safety of AAV-based therapies in the context of WD.

P0885

Development of *in vivo* genome editing for the treatment of progressive familial intrahepatic cholestasis type 2

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Progressive familial intrahepatic cholestasis type 2 (PFIC-2) is a monogenic inborn error of metabolism caused by the deficiency of the liver bile salt export pump (BSEP), the transporter of conjugated bile acids (BA) across the canalicular membrane of hepatocytes, encoded by *Abcb11*. PFIC-2 patients display cholestasis, jaundice, itching, and usually develop liver fibrosis and end-stage liver disease before adulthood. Current treatments consist of diet control, and pharmacological or surgical interventions aiming at decreasing BA blood concentration. However, most patients ultimately undergo liver transplantation as the only curative option. In diseases like PFIC-2, presenting with early onset and rapid disease progression, early therapeutic intervention would be fundamental to limit progressive damage to hepatocytes. Since BSEP expression is tightly regulated by BA levels through the farnesoid-X-receptor signalling, acting on *Abcb11* endogenous promoter, we are developing a liver-directed CRISPR-Cas9 based-genome editing approach aimed at integrating a corrective cDNA into the first intron of the *Abcb11* gene. This strategy may allow for correcting most of the disease-causing mutations while maintaining as much as possible the physiological gene regulation. Moreover, this approach may enable stable genetic correction even in pediatric patients at the first disease stages. We initially assessed LV-mediated liver gene addition in *Abcb11*$^{-/-}$ mice, a PFIC-2 model, to correlate therapeutic effects with the extent of liver modification. We administered 2-week-old *Abcb11*$^{-/-}$ mice with LV encoding the murine BSEP transgene under the control of a hepatocyte-specific cassette based on the enhanced transthyretin promoter. We observed that transgene mRNA levels of about 30% of the endogenous murine BSEP resulted in a significant decrease, up to normalization, of serum BA, bilirubin, and alkaline phosphatase biomarkers until 6 months of age in both males and females, indicating prevention of cholestatic damage. Towards genome editing, we selected the most efficient *Abcb11*-targeting guide RNA (gRNA) in hepatocyte cell lines. Moreover, we compared different mCherry-expressing donor DNAs designed to exploit homology-directed repair (HDR) or homology-independent targeted integration (HITI) pathways for site-specific integration in the first intron of *Abcb11* gene. We achieved the highest integration rate when using the HDR-based donor DNA (up to 12%). *In vivo*, we tested the different mCherry-donor DNA configurations delivered via adeno-associated viral vectors (AAVs) to 2-day or 2-week-old Cas9 transgenic mice. In the liver of mice of both ages, we observed a slightly higher integration rate (up to 12%), mCherry expression, and mCherry-positive liver area (up to 14%) exploiting homology-dependent rather than -independent pathway. The treatment of 2-week-old wild-type mice with AAV-donor DNA, followed by administration of lipid nanoparticles carrying Cas9 mRNA and gRNA, confirmed higher efficiency of the HDR-based donor construct compared to HITI. We are currently testing the selected editing reagents in *Abcb11*$^{-/-}$ mice to evaluate both the liver site-specific integration rate and therapeutic efficacy

in the mouse model of PFIC-2, treated early in life. Overall, these studies will inform about the feasibility and efficacy of *in vivo* liver-directed genome editing for the treatment of PFIC-2.

P0886

Hematopoietic Stem Cell Gene Therapy for Hurler Syndrome (OTL-203): interim skeletal, neurological and systemic outcomes

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Preliminary results of an ongoing phase I/II study of OTL-203, an autologous hematopoietic stem gene therapy (HSC-GT, NCT03488394), in 8 Mucopolysaccharidosis type I Hurler syndrome (MPSIH) patients suggest superior metabolic correction and initial clinical response compared to allogeneic haematopoietic stem cell transplantation (HSCT) published in literature (Gentner *et al. N Engl J Med* 2021; *Consiglieri et al. Sci Transl Med 2024*). Here we report interim skeletal, neurological and systemic outcomes up to 4-year after HSC-GT.

IDUA activity was measured in blood and cerebral spinal fluid (CSF), dermatan (DS) and heparan sulphate (HS) in urine and CSF at baseline and different timepoints up to 4-year after OTL-203 treatment. Skeletal, neurological, corneal clouding (CC), carpal tunnel syndrome (CTS) and cardiac outcomes were assessed at baseline and different timepoints up to 4-year after treatment. For specific outcomes OTL-203 patients were compared with an external cohort of MPSIH patients who underwent HSCT.

IDUA activity in blood reached supraphysiologic levels in all patients from +30 days after OTL-203 treatment resulting in urinary substrate clearance, maintained at last follow-up. All patients exhibited longitudinal growth within expected reference ranges according to age and gender with a median height gain greater than HSCT patients and experienced an earlier normalization of joint mobility compared with HSCT patients. Both hip X-ray and MRI parameters improved after OTL-203 treatment, underlining an amelioration of hip dysplasia up to last follow-up. A specific spine MRI score showed stabilization of the typical spine MRI features at last follow-up. From 3 months after OTL-203 treatment, IDUA activity became detectable in CSF and persisted overtime accompanied by local reduction of DS/HS (undetectable DS in 7/8 patients at +4-year follow-up). After OTL-203 treatment, 7/8 patients continued to gain motor and cognitive skills in line with typically developing children and experienced an improvement of typical brain MRI abnormalities. At baseline, all subjects had evidence of mild to moderate CC. Of the OTL-203 patients, 3/8 achieved CC resolution and 5/8 had mild CC at last follow-up. In the HSCT cohort, no patient experienced CC resolution with all patients showing moderate CC at 5-year after HSCT. A specific echocardiac score showed worsening of cardiac involvement in HSCT patients (92.5%) compared to OTL-203 patients who stabilized or improved their score. Throughout the follow-up window, the median cardiac score of OTL-203 patients was significantly lower than HSCT ($p<0.001$). One OTL-203 patient underwent carpal tunnel surgery before treatment and another 6 months after treatment due to pre-existing severe damage. The remaining patients did not develop CTS. In the HSCT cohort 7/9 patients developed CTS, requiring surgery at a median of 3.7 years post-HSCT.

This interim analysis indicates an early beneficial effect of OTL-203 on MPSIH-typical skeletal, neurological, CC, cardiac and CTS manifestations up to 4-year after treatment, together with biochemical detoxification in urine and CSF. A randomized multicenter Phase III trial (NCT06149403) sponsored by Orchard Therapeutics evaluating the safety and efficacy of autologous HSC-GT OTL-203 compared to allo-HSCT is in progress.

P0887

Investigating Busulfan's effect on the Blood-Brain Barrier to enhance CNS engraftment in hematopoietic stem cell gene therapy for neurometabolic disorders

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Neurological lysosomal storage disorders (LSDs) are metabolic diseases caused by dysfunctional enzymes leading to toxic accumulation of macromolecules, especially within the central nervous system (CNS). Haematopoietic stem cell (HSC) gene therapy has shown potential in alleviating neurological symptoms and preventing further neurodegeneration, primarily through the CNS engraftment of HSC-derived microglial-like cells. However, the treatment's success depends on the HSC's ability to cross the blood-brain barrier (BBB). Experimental and clinical evidence indicates that the conditioning agent busulfan, used prior to transplantation to clear the bone marrow niche, can enhance HSC engraftment in the CNS, though the underlying mechanisms are not fully understood. Beyond its critical role in depleting resident microglia, prior research suggests that busulfan may also cause vascular injury and potentially involving BBB disruption. We hypothesize that a disrupted BBB may partly contribute to improved HSC engraftment. To this end, we conducted in vivo transplantation studies of lin- murine HSCs in mice conditioned with either busulfan or irradiation, finding that busulfan increases HSC engraftment exclusively to the CNS, and not to other tissues. This suggests that busulfan's mechanism(s) of action is CNS-specific and may involve BBB remodelling. Additionally, we performed comprehensive immunofluorescent imaging and quantification of CNS microvasculature in vivo at various time points post-conditioning. We specifically assessed both brain microvasculature structure and brain endothelial tight junction integrity and organization. Although we did not observe acute macroscopic brain vascular injury or gross disruption of the BBB structure in busulfan-treated mice compared to irradiated ones, we found a significant downregulation of Claudin-5, a key brain endothelial tight junction protein highly expressed in the BBB, in busulfan-treated mice. Claudin-5 downregulation has been previously shown to be associated with increased BBB permeability, suggesting that a subtle and rapid, but reversible, BBB remodelling may occur following busulfan treatment. These findings offer new insights into the effects of pre-conditioning regimes on CNS vasculature, paving the way for new investigations into BBB manipulation to increase permeability and thereby enhance the therapeutic efficacy of HSC based gene therapy for neurometabolic disorders.

P0888

Liver-directed gene therapy normalizes toxic bile acid metabolite levels in the blood and brain of mice with cerebrotendinous xanthomatosis

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Cerebrotendinous xanthomatosis (CTX) is an autosomal recessive disease caused by mutations in the *CYP27A1* gene, that encodes for the sterol 27-hydroxylase, essential for the conversion of cholesterol to bile acids. Loss-of-function mutations in *CYP27A1* lead to the accumulation of toxic precursors, such as 7 alpha-hydroxy-4-cholesten-3-one (7αC4) and cholestanol, in blood, eyes, tendons and brain, resulting in the formation of cataracts, xanthomas and neurodegeneration. The standard treatment is chenodeoxycholic acid, which reduces the levels of toxic metabolites but does not always prevent neurodegeneration leaving an unmet medical need. Liver-directed gene therapy is a valuable alternative for CTX patients, since published data showed a correction of disease biomarkers upon *CYP27A1* gene supplementation using an adeno-associated virus (AAV) serotype 8 in CTX mice. To develop an AAV vector with high potency and low immunogenicity, several optimized *CYP27A1* expression cassettes containing a liver-specific promoter were designed with a) three codon-optimized transgene sequences depleted of CpG motifs to avoid the potential activation of an innate immune response and b) addition of regulatory elements: minute virus of mice small intron (MVM) and Woodchuck Hepatitis Virus post-transcriptional regulatory element (WPRE). Transfection of Huh7 cells with the different plasmids allowed to select the best two candidates. Next, the therapeutic efficacy of the selected candidates AAV8-CYP27A1co1 and AAV8-MVM-CYP27A1co1-WPRE was tested at different doses and compared to an AAV8 vector carrying the WT sequence of *CYP27A1*, in CTX mice. Inclusion of the regulatory elements to enhance expression did not bring any benefit *in vivo*. However, both *in vitro* and *in vivo* results showed higher CYP27A1 expression with VTX-806(8), candidate bearing the codon-optimized version of the transgene. Treatment with VTX-806(8) restored normal levels of circulating 7αC4, expression of hepatic bile acid synthesis-related genes and corrected hepatomegaly. Most importantly, normal levels of 7αC4 in the brain of CTX mice were restored. These results showed for the first time that liver-directed AAV gene therapy can normalize the levels of toxic metabolites in the brain of CTX mice and thus may offer an effective curative treatment option for CTX patients.

P0889

Pre-clinical development of an *ex-vivo* Hematopoietic Stem/progenitor Cells-gene therapy for α-Mannosidosis

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α-Mannosidosis (α-MAN) is a rare lysosomal storage disorder (LSD) due to mutations in the MAN2B1 gene encoding for α-D-mannosidase (MAN2B). MAN2B inactivity leads to accumulation of undigested mannose-enriched oligosaccharides in the lysosomes, with progressive skeletal and organ damage, immune deficiency, hearing impairment and intellectual disability. Enzyme replacement therapy and allogeneic hematopoietic stem/progenitor cells (HSPC) transplantation show partial improvement of metabolic alterations but low efficiency in correcting the skeletal manifestations. *Ex-vivo* lentiviral (LV)-based HSPC-gene therapy (GT) proved its efficacy in correcting the clinical phenotype of other LSDs restoring the enzymatic activity also in non-hematopoietic tissues (e.g. central nervous system and bones) through cross-correction. Indeed gene-corrected tissue-resident hematopoietic cells are capable of releasing supra-physiological level of the deficient enzyme that is uptaken by neighbor cells of non-hematopoietic origin, restoring their function. Based on these results, we developed lentiviral vectors encoding for the wild-type (WT) form as well as the codon optimized (OPT) sequence for MAN2B for the treatment of α-Mannosidosis, as part of a GT platform program for LSDs with skeletal involvement.

Our data showed that healthy donor (HD)-derived CD34+ cells transduced with WT and OPT vectors retain their clonogenic and proliferation capability *in vitro*. LV-MAN2B transduced CD34+ cells, as well as their differentiated osteoclasts, showed supra-physiological intracellular and extracellular enzymatic activity as compared to untransduced control. The released enzyme was also capable to restore MAN2B activity in α-MAN patients' fibroblasts at levels comparable to HD-fibroblasts. MAN2B-transduced HD CD34+ cells were also xeno-transplanted in immunodeficient mice displaying multilineage reconstitution and 3-fold higher enzyme activity in the bone marrow of transplanted mice than mock controls. Moreover, lineage negative (Lin-) murine cells, transduced with LV-MAN2B at different MOIs, were exploited to perform *in vitro* and *in vivo* tox studies. LV- MAN2B transduced Lin- cells displayed no toxicity *in vitro* and increased intracellular and extracellular enzymatic activity versus untransduced cells. Additionally, MAN2B overexpression was well tolerated by murine HSPC upon transplantation, showing retention of their engraftment and differentiation capability. Finally, we are currently characterizing the skeletal and immunological phenotype of a *MAN2B1-/-* mouse model, that will

be instrumental for the proof-of-concept studies for α-MAN HSPC-GT. Preliminary tests on 4-months-old *MAN2B1-/-* mice showed alterations in the length of long bones and bone mineralization, associated with reduced motor coordination.

Altogether, our data support the development of an *ex-vivo* LV-based HSPC-GT approach for the treatment of α-Mannosidosis, also providing a comprehensive characterization of the pre-clinical model for testing the HSPC-GT efficacy.

P0890

Dose-finding and toxicological studies of intravenous administration of an AAV9-*ASAH1* vector for Farber disease and spinal muscular atrophy with progressive epilepsy

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Farber disease (FD) and spinal muscular atrophy with progressive myoclonic epilepsy (SMA-PME) are autosomal recessive disorders caused by mutations in the *ASAH1* gene, which codes for acid ceramidase (ACDase), a lysosomal enzyme that catalyses the bioactive lipid ceramide into sphingosine and fatty acid. These *ASAH1*-related disorders present with a spectrum of clinical manifestations during early (Farber disease, FD) or late infancy/teenage (spinal muscular atrophy with progressive myoclonic epilepsy, SMA-PME). To date, there is no curative treatment for patients and therefore a clear unmet medical need. We established proof-of-concept studies that intravenous administration of a recombinant AAV9 vector expressing human ACDase in *Asah1*$^{P361R/P361R}$ mice at post-symptomatic stages of the disease is able to prolong the lifespan and correct the phenotype. Here, we performed a dose escalation study in *Asah1*$^{P361R/P361R}$ mice to determine the minimum effective dose (MED) of this vector. Three doses of AAV9-*ASAH1* were administrated intravenously in mutant mice at a late stage of the disease and the effect was analyzed at the clinical, molecular and histological level for a 6-month period. We found that the low dose was suboptimal, whereas most pathological parameters were corrected at mid dose. However, detailed histological analysis of treated mice 6 months post-injection revealed that administration of the vector at the high dose was able to avoid the presence of inflammatory infiltrates in some tissues, including the central nervous system. Based on these results, we performed a 3-month toxicological study in rats with AAV9-*ASAH1* administrated intravenously at two doses (MED and 7x MED), and findings will be presented. Altogether, these results paved the way for a compassionate use of the vector in a SMA-PME patient.

P0891

Five new murine models of PCCB deficiency created by transgenesis and gene editing recapitulate the genetic diversity and clinical spectrum of propionic acidemia and enable the first successful proof-of-principial gene therapy studies

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Propionic acidemia (PA) is rare autosomal recessive metabolic disorder caused by defects in the mitochondrial localized enzyme propionyl-CoA carboxylase (PCC). The PCC enzyme is composed of six α- and six β-subunits with causative variants occurring at equal frequencies in either the nuclear encoded *PCCA* or *PCCB* gene. Individuals with PA can suffer from poor growth, potentially lethal metabolic decompensations, and cardiomyopathy despite current medical management, which has led to the pursuit of gene therapy as a new treatment option for patients. There are currently no animal models of PA caused by PCCB deficiency. To explore the pathophysiology and to test new therapies for PA, we have developed 5 new murine models of PCCB deficiency. CRISPR-Cas9 gene editing of the murine *Pccb* gene was used to create loss of function alleles: an 8 bp deletion in the 7th exon, and a 4 bp deletion in the 14th exon. Both alleles result in a frameshift and premature stop codon in the *Pccb* gene. $Pccb^{E7_8bpdel/E7_8bpdel}$ and $Pccb^{E14_4bpdel/E14_4bpdel}$ homozygotes manifest an immediate neonatal lethal phenotype accompanied by massive elevations of disease related metabolites, 2-methycitrate (2-MC) and 3-hydroxypropionate (3-OHP). Due to the severe lethality displayed by the homozygous *Pccb* knock-out mice, we designed a germline transgene to express the murine *Pccb* cDNA under the control of a muscle specific promoter ($Tg^{Mck\text{-}Pccb}$) and bred to rescue the $Pccb^{E14_4bpdel/E14_4bpdel}$ mice. The resultant $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{Mck\text{-}Pccb}$ animals are rescued from neonatal lethality but perish by 2 months of age. $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{Mck\text{-}Pccb}$ mice have milder biochemical perturbations of plasma 2-MC and 3-OHP in comparison to the $Pccb^{E14_4bpdel/E14_4bpdel}$ mice but are severely growth retarded, very much like patients with PA, and only achieve ~ 50% of the weight of unaffected littermates at weaning. To generate mice with a late onset form of PA, we used CRISPR-Cas9 gene editing to introduce the c.C689T (p.P230L) mutation into the murine *Pccb* gene. PA patients who are homozygous for the orthologous missense mutation in the human *PCCB* c.C683T (p.P228L) are mildly affected, but symptomatic when stressed. $Pccb^{P230L/P230L}$ homozygous were bred to $Pccb^{WT/\ E7_8bpdel}$ mice to create compound heterozygous $Pccb^{P230L/\ E7_8bpdel}$ mice. Both $Pccb^{P230L/P230L}$ and $Pccb^{P230L/\ E7_8bpdel}$ mice display attenuated metabolic changes and less impaired growth than $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{Mck\text{-}Pccb}$ mice but experience lethal metabolic decompensation when fed an increased protein diet. To demonstrate the utility of these models to test new therapies, $Pccb^{E14_4bpdel/E14_4bpdel}$ were treated with AAV9.CAG.PCCB at a dose of 1e11 vg/pup at birth, which resulted in a significant increase in survival in comparison to untreated mutant controls (p<0.01). We also treated $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{Mck\text{-}Pccb}$ mice at weaning with a dose of 1e14 vg/kg with either AAV9.CAG.PCCB or AAV9.EF1a.PCCB vectors, which likewise resulted in a significant increase in survival and growth in comparison to untreated $Pccb^{E14_4bpdel/E14_4bpdel}$;$Tg^{Mck\text{-}Pccb}$ controls . These new murine models replicate the clinical and biochemical features of PA over wide clinical spectrum, spanning neonatal, juvenile, and late onset disease presentations, and can be used to test the effects of new genomic and small molecule therapies for PA caused by PCCB deficiency.

P0892

Liver fibrosis negatively impacts *in vivo* gene transfer to hepatocytes

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Liver fibrosis is a pathological process occurring in several genetic and acquired disease conditions. The damage induced by fibrosis has an impact on liver morphology and on the metabolism of hepatocytes, which may negatively affect therapeutic approaches targeting the liver. Among these, adeno-associated viral (AAV) vectors demonstrated clinical efficacy and safety for many inherited diseases. An alternative is represented by integrating lentiviral vectors (LVs) that have been shown to allow for stable long-term transgene expression in preclinical models of liver disorders. Recently, lipid nanoparticles (LNPs) have emerged as promising non-viral vehicles to deliver RNA-based therapeutics or genome-editing agents to the liver. Here, we assessed the impact of pre-existing liver fibrosis on hepatocyte gene transfer mediated by AAV vectors, LVs, or LNPs. Since different insults cause different types of liver fibrosis, we took advantage of two representative chemically induced mouse models. The first model is generated by repetitive administrations of carbon tetrachloride (CCl_4) for 3 or 6 weeks, causing intermediate or severe toxic fibrosis localized in the hepatic pericentral area. The second model is based on a diet supplemented with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) for 3 or 5 weeks, which leads to intermediate or severe biliary fibrosis localized in the hepatic periportal area. We exploited CCl_4- and DDC to induce liver fibrosis in either wild-type (WT) or floxed-tdTomato reporter mice and confirmed the fibrotic state of the liver. We then intravenously administered LVs or AAV vectors encoding for reporter genes (human factor IX or mCherry) to WT mice, or we delivered LNPs carrying the CRE-recombinase mRNA to floxed-tdTomato mice. We observed that both intermediate and severe pericentral and periportal fibrosis negatively affected LV-mediated hepatocyte gene transfer (4-fold reduction) and LNP-mediated RNA delivery (2-fold reduction). On the contrary, the efficiency of AAV-vector-mediated transduction was reduced just in case of preexisting intermediate or severe periportal fibrosis (2-fold reduction). Moreover, we employed *Abcb11*$^{-/-}$ and *Agl*$^{-/-}$ mice, which recapitulate features of progressive familial intrahepatic cholestasis type 2 and glycogen storage disease type III, as representative models of genetic disorders characterized by liver fibrosis. We observed mild and minimal periportal fibrosis in the liver of 6-month-old *Abcb11*$^{-/-}$ mice and 9-month-old *Agl*$^{-/-}$ mice, respectively. We administered them with LV or AAV vectors encoding for mCherry. Gene transfer by both LV and AAV vectors was slightly reduced in *Abcb11*$^{-/-}$ mice, while it was not altered in *Agl*$^{-/-}$ mice. These data indicate an inverse correlation between the severity of liver fibrosis and the transduction efficiency of hepatocytes by viral vectors. This work provides evidence that significant hepatic fibrosis generally reduced transduction and transfection efficiency of hepatocytes, with different outcomes according to the vector used and the state of the liver at the time of vector administration. Overall, this study has relevant implications for developing liver-directed gene addition or genome editing approaches and highlights the importance of evaluating liver fibrosis as inclusion/exclusion criteria when treating patients with gene therapies targeting the liver.

P0893

Long-term correction of acid ceramidase deficiency by intravenous AAV-mediated gene therapy in presymptomatic P361R-Farber mice

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Acid ceramidase (ACDase) is a ubiquitously expressed lysosomal enzyme that catabolizes ceramide into sphingosine and free fatty acid. Acid ceramidase deficiency caused by mutations in the *ASAH1* gene leads to Farber disease (FD) and spinal muscular atrophy with progressive myoclonic epilepsy (SMA-PME), two ultra-rare lysosomal storage disorders (LSD) in which ceramides accumulate in cells and lead to the presence of inflammatory infiltrates and lesions in various tissues. Patients with the most severe forms of FD die in infancy and suffer from hepatosplenomegaly, along with haematological, respiratory, and neurological alterations. In general, SMA-PME patients present with progressive lower motor deficits and myoclonic epilepsy and succumb to the disorder in late childhood or early adulthood. There is a clear unmet medical need due to the lack of effective therapies. Here, using a severe mouse model of Farber disease (*Asah1*$^{P361R/P361R}$, P361R-Farber), we tested the effect of systemic delivery of recombinant adeno-associated vector 9 (rAAV9) expressing *ASAH1* coding sequence on long-term prevention of the disease. Presymptomatic mutant mice (3-week-old) were intravenously injected with AAV9-*ASAH1* or saline solution and analyzed at the clinical, molecular, and histological level for a 1-year period. FD mice injected with saline solution died by 8-12 weeks of age, whereas 90% of AAV9-*ASAH1*–treated FD mice survived until 56 weeks of age with a correction in body growth, respiratory function and marked reduction of inflammation in peripheral tissues and the central nervous system. Of note, at 56 weeks, 3 of 10 treated FD mice exhibited benign liver tumours. Our results establish the proof-of-concept that systemic AAV-mediated *ASAH1* gene therapy is efficacious for long-term correction of Farber disease/SMA-PME in mice.

P0894

Rescue of glutaric aciduria type I mice by liver directed therapies

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Glutaric Aciduria Type I (GA-1) is an inborn error of metabolism with a severe neurological phenotype caused by the deficiency of Glutaryl-CoA dehydrogenase (*GCDH*), the last enzyme of lysine catabolism. Using the murine GA-1 model (*Gcdh*$^{-/-}$ mouse), we recently elucidated that toxic GA-1 catabolites in the brain originate from the liver. Our findings uncovered the liver as a more accessible target organ than the brain for a therapeutic approach for GA-1. Moreover, we demonstrated that the characteristic brain and lethal phenotype of the mouse model can be

rescued by two different liver directed gene therapy approaches. Here, we will share our ongoing therapeutic approaches (gene editing & nucleic acid therapeutics) and discuss chances and challenges.

P0895

Preclinical development of hematopoietic stem cell-gene therapy for Mucopolysaccharidosis type IVB using a GLB1 transgene with enhanced therapeutic potential

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Mucopolysaccharidosis type IVB (MPSIVB) is a lysosomal storage disorder (LSD) caused by b-GAL deficiency (b-GAL), a lysosomal enzyme encoded by the GLB1 gene. Depending on the pathogenic variants of the GLB1 gene, patients may develop a purely skeletal disease (MPS IVB or Morquio syndrome B) or may also exhibit rapidly progressive neurodegeneration (GM1 gangliosidosis) (1). Currently, there are no approved therapies for these patients. However, clinical data obtained by our Institute have demonstrated the safety and efficacy of Hematopoietic Stem and Progenitor Cell-Gene Therapy (HSPC-GT) in other forms of LSDs. These findings suggest that the gene-corrected HSPC progeny releases supraphysiological levels of the therapeutic enzyme, which can effectively target both brain and skeletal tissues (2-4). Based on these results, we aim to develop an ex-vivo GT approach for patients with MPSIVB and GM1 gangliosidosis. We designed lentiviral vectors (LVs) encoding human b-GAL to achieve supraphysiological release of the therapeutic enzyme in human HSPCs and metabolic correction of diseased cells. Transduced human HSPCs displayed normal colony formation, proliferation, and differentiation capacity in vitro and in vivo. However, despite overexpressing b-GAL intracellularly, the progeny of transduced HSPCs failed to release the enzyme at supraphysiological levels. Therefore, we tested alternative LVs to overexpress an enhanced b-GAL deriving from murine (LV-enhGLB1) and human selectively mutated GLB1 sequences (LV-mutGLB1). HSPCs transduced with LV-enhGLB1 overexpressed b-GAL in vitro and after transplantation in immunodeficient mice without evidence of b-GAL overexpression-related toxicity. Myeloid cells and osteoclasts derived from LV-enhGLB1 transduced HSPCs, but not from the modified human enzyme, released b-GAL at supraphysiological levels, allowing the cross-correction of defective cells, including skeletal cells. We found that the cell-specific human GLB1 mRNA half-life and the improved stability of the enhanced b-GAL contribute to the increased efficacy of LV-enhGLB1. Importantly, the enhanced b-GAL enzyme showed physiological lysosomal trafficking in human cells and was not associated

with increased immunogenicity in vitro. These results support the use of LV-enhGLB1 for further HSPC-GT development and future clinical translation to treat MPSIVB multisystem disease.

P0896

In utero base editing using virus-like particles in a mouse model of Krabbe disease

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Infantile Krabbe disease (KD) is a rare neurological disorder caused by a deficiency in the lysosomal enzyme β-galactocerebrosidase (GALC), leading to demyelination and neuroinflammation of the central and peripheral nervous systems (CNS and PNS). Without treatment, KD results in progressive neurodegeneration leading to early death. Currently, there is no definitive treatment for KD. Hematopoietic stem cell transplantation only delays disease progression by reducing neuroinflammation through healthy hematopoietic stem cell-derived myeloid cells, and only if performed in pre-symptomatic newborns, highlighting the tight therapeutic window. Therefore, there is an urgent need for a safe and effective therapy capable of correcting the underlying genetic mutations, preventing early-onset neuropathy, and preserving brain development in affected infants.

To address this medical need, we are developing a prenatal in vivo gene editing platform to correct GALC mutations in the CNS, PNS, and hematopoietic system using adenine base editors (ABEs). In utero (IU) intervention via intravenous injection offers numerous advantages, including early treatment prior to disease onset, improved accessibility to the brain and to proliferating hematopoietic progenitors in the liver, increased immune tolerance, and reduced doses of ABEs needed. To test the therapeutic potential of ABEs in the context of KD we utilize a Twitcher (TWI) mouse model. TWI mice display a nonsense mutation (TGA>TGG), that induces a premature stop codon in the *Galc* gene, leading to a lack of functional GALC enzyme and clinical symptoms of severe KD. ABEs and the corresponding guide RNA targeting the point mutation are delivered via Virus Like Particles (VLPs) which guarantee rapid, transient, and widespread delivery to various cell types implicated in KD. We assessed the efficiency of ABE-eVLPs in Schwann cells and hematopoietic progenitors derived from TWI mice, achieving up to 95% base editing in dose escalating experiments with different ABE versions. Base editing restored *Galc* reading frame, leading to the reconstitution of GALC protein expression, processing and function at levels comparable to that detected in healthy cells. When comparing ABE8e-NG versus ABE7.10-NG, the latter resulted in comparable editing efficiency but lower bystander edits that did not alter GALC protein structure and function, as confirmed by bioinformatic prediction and by functional assays. Gene correction also neutralized KD typical signs of neuroinflammation, thus demonstrating the high efficiency of the gene correction platform. IU delivery of reporter VLPs (YFP-VLPs) injected into mice via the vitelline vein demonstrated increased targeting of foetal hematopoietic progenitors as well as hematopoietic stem cells in the liver. Critically, we could also detect YFP expression in the brain suggesting VLPs crossing the permeable foetal blood brain barrier. We are now testing the ability of our platform to provide gene correction as well as functional GALC restoration in the relevant tissues by IU and perinatal delivery of ABE7.10-NG-

VLPs to TWI foetuses and newborn pups. Our goal is to create a safe and efficient in vivo base editing platform to treat KD by targeting the hematopoietic system and the brain simultaneously before disease onset, potentially serving as a proof-of-concept for other early-onset lysosomal storage disorders and neurodegenerative diseases.

P0897

Adeno-Associated Virus-Based Gene Therapy Delivering Dual Growth-Associated Genes to MPS IVA Mice

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Mucopolysaccharidosis type IVA (MPS IVA) results from a deficiency in the enzyme galactosamine (N-acetyl)-6-sulfatase (GALNS), which is crucial for breaking down certain glycosaminoglycans (GAGs). The continuous build-up of GAGs causes various skeletal issues (such as short stature, hypoplasia, and tracheal obstruction) and affects other organs. Currently, there is no effective treatment for bone abnormalities in these patients. To address this, we propose an innovative combination therapy using adeno-associated virus (AAV) vectors to deliver the GALNS enzyme and a natriuretic peptide C (CNP, encoded by the NPPC gene) to promote growth in MPS IVA. In our study, we treated an MPS IVA mouse model with an AAV vector expressing the GALNS enzyme in conjunction with another AAV vector expressing the NPPC gene, monitoring them for 12 weeks. The combination therapy led to increased enzyme activity in various tissues (including bone, liver, heart, and lung) and in plasma, resulting in enhanced bone growth. Additionally, significant improvements in bone morphology were observed in mice treated with CNP, which corresponded with higher CNP activity in plasma. Our findings suggest that combining CNP with GALNS gene therapy boosts bone growth in MPS IVA mice more effectively than GALNS gene therapy alone. This combination approach could be a promising alternative, given that enzyme therapy by itself does not adequately target the bone growth regions.

P0898

Unveiling myeloid-mediated enzymatic correction of ARSA-deficient neural cells in hematopoietic stem cell gene therapy for Metachromatic Leukodystrophy

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Metachromatic Leukodystrophy (MLD) is an autosomal recessive lysosomal storage disease caused by deficiency of Arylsufatase A (ARSA), a critical enzyme that breaks down sulfatides. Accumulation of sulfatides results in neurological manifestations related to white matter loss in the central and peripheral nervous systems (CNS, PNS), accompanied by neuroinflammation and neurodegeneration.

Ex vivo hematopoietic stem cell gene therapy (HSC-GT) using autologous HSCs engineered by lentiviral vectors (LV) to express supraphysiological ARSA levels is the only approved treatment for MLD. More than ten years of robust safety and efficacy data culminated in the full marketing authorization of this HSC-GT approach for MLD (atidarsagene autotemcel, "arsa-cel") in Europe, UK and USA. This groundbreaking one-time gene therapy treatment is indicated for children with pre-symptomatic late infantile, pre-symptomatic early juvenile or early symptomatic early juvenile MLD subtypes. While it is acknowledged that engraftment of metabolically proficient HSC myeloid progeny in CNS tissues play an indispensable role in immunomodulation and neuroprotection post-treatment, the precise mechanism of myeloid-mediated enzymatic cross-correction of MLD neural cells is debated. Therefore, we have explored myeloid-mediated ARSA cross-correction mechanisms using relevant *in vitro* human models.

Our research unveiled that ARSA-overexpressing MLD human monocyte-derived macrophages actively release a functional ARSA enzyme, effectively correcting ARSA-deficient neurons and glial cells derived from patient-specific induced pluripotent stem cells (iPSCs). ARSA overexpression did not affect the M1/M2 macrophage polarization, which did not influence ARSA secretion from myeloid cells. Furthermore, transgenic ARSA released by LV.ARSA-transduced MLD macrophages and myeloid cells purified from HSC-GT-treated MLD patients efficiently cross-correct MLD hiPSC-derived neurons and glia. Of note, ARSA released in the cerebrospinal fluid of healthy donors retains similar cross-correction properties. Delving deeper into the mechanism of myeloid-to-neural enzymatic cross-correction, we showed that transgenic ARSA enzyme is post-transcriptionally modified through the phosphorylation of mannose-6 residues and its uptake by MLD neural cells is partly mediated by the mannose-6-phosphate receptor.

Our findings underscore the consistent occurrence of myeloid-mediated enzymatic cross-correction of ARSA-deficient neurons and glial cells within a clinically relevant HSC-GT framework, offering profound insights into the therapeutic potential of this approach for genetic neurological diseases.

P0899

Towards modeling human MODY1 in mice

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MODY1 is the third-most common subtype of Maturity-Onset Diabetes of the Young, a rare form of diabetes accounting for about 1-2% of all cases. MODY1 is caused by mutations in the transcription factor HNF4A and leads in humans to diabetic complications at similar rates as type 1 or type 2 diabetes with insulin therapy often being required. To explore gene therapy as a cure for monogenic diabetes and better understand the progression of the disease, we have created eight different mouse models for human MODY1. The generated mouse models range from (*i*) whole-body allele deletions over (*ii*) the point mutations *Hnf4a*^{G124A} and *Hnf4a*^{R333L}, which also occur in humans, to (*iii*) a beta cell specific downregulation of HNF4A expression in pancreatic islets by a knockin of two mircoRNA-375 binding sites into the 3'UTR of the gene, creating the mouse line *Hnf4a*^{mirT375}. Starting at 6 weeks of age, the experimental pipeline included weekly body weight and biweekly blood glucose measurements, intraperitoneal glucose tolerance tests (i.p.GTT) at 8 and 20 weeks of age, and intraperitoneal insulin tolerance tests (i.p.ITT) at 12 and 22 weeks of age (n = 12 - 15). Plasma insulin levels were determined at multiple time points. Pancreatic islets were isolated post mortem for qRT-PCR analysis (n = 4). In an additional experiment, the mouse line *Hnf4a*^{mirT375} was exposed to a metabolic challenge in the form of a high-fat diet over a period of 8 weeks in the experimental pipeline (n = 12 - 15). Whole-body HNF4A manipulations (allele deletion and point mutations) showed either prenatal lethality in homozygotes or no diabetic phenotype. Employing the knockin method, we were able to achieve a > 80% reduction in *Hnf4a* mRNA levels in isolated pancreatic islets ($p < 0.0001$). However, a diabetic phenotype could not be detected by routine blood glucose measurements ($p = 0.771$), glucose (i.p.GTT at 20 weeks of age: $p = 0.874$) and insulin response assessments (i.p.ITT at 22 weeks of age: $p = 0.816$). Plasma insulin levels at 6, 14 and 24 weeks of age showed no difference between groups with p-values between 0.146 to 0.207. The metabolic challenge of a high-fat diet also did not result in a diabetic phenotype. An expression profile analysis on isolated pancreatic islets supports this finding. Despite employing various techniques, we could not yet succeed in generating a suitable model for an established human disease. Our work indicates substantial differences between mouse and human HNF4A function in the pancreas. When testing new strategies for MODY1 modeling in mice, these aspects need to be taken into account. This study may serve as a reminder that findings generated in mice cannot always be easily translated to humans.

P0900

AAV-mediated liver-directed gene therapy for maple syrup urine disease due to E2 defects

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Maple syrup urine disease (MSUD) is an inherited metabolic disorder caused by deficiency of the branched-chain-ketoacid dehydrogenase (BCKDH) complex, involved in the breakdown of branched-chain amino acids (BCAA). The inner-mitochondrial enzyme BCKDH complex consists of three catalytic components: the branched-chain alpha-ketoacid decarboxylase (made of E1α and E1β), the dihydrolipoyl transacylase (E2) and the dihydrolipoamide dehydrogenase (E3). Pathogenic variants in any of the genes encoding the components of the complex result in MSUD. Current therapy of MSUD is based on dietary restrictions of BCAA or liver transplantation. AAV-mediated gene therapy holds promise as non-invasive and more effective treatment of MSUD. We generated a new mouse model of MSUD harboring a variant changing histidine with arginine at amino acid position 452 in the E2 component of the murine BCKDH. In humans the

corresponding pathogenic variants in the E2 enzyme result in deficiency of BCKDH that is responsive to thiamine. Mice homozygous for this variant (*E2*$^{H452R/H452R}$) showed increased perinatal mortality with 100% of mice dying within the first 10 days of life, reduced body weight, and increased concentrations of BCAA in blood. Homozygous *E2*$^{H452R/H452R}$ mice were intraperitoneally injected with 100 mg/kg of Thiamin-HCl starting from postnatal day 1 that resulted in mild increase in survival. To investigate the efficacy of liver-directed gene therapy, we injected *E2*$^{H452R/H452R}$ pups in the temporal vein on day one of life with a AAV8 vector expressing the human E2 coding sequence under the control of the liver-specific Thyroxine-binding globulin (TBG). Following vector administration *E2*$^{H452R/H452R}$ pups showed extended survival and recovery in body weight up to 20 days of age. Consistent with previous studies, increased mortality and loss of transgene expression was detected from day 20 of life as consequence of hepatocyte replication and vector genome dilution. In conclusion, we showed that gene replacement by AAV rescued the perinatal lethality of severe MSUD due to E2 defect. Approaches based on genome integration of the therapeutic gene have the potential to provide long-term phenotypic correction in severe MSUD.

P0901

Characterization of a mouse model for MODY4: insights from phenotypic analysis

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Maturity-Onset Diabetes of the Young (MODY) is a rare form of diabetes and accounts for approximately 1-2% of all cases. MODY4 is caused by mutations in the *PDX1* gene, which plays a crucial role in pancreatic development and regulation of insulin production and secretion. Because this disease is often misdiagnosed as type 1 or type 2 diabetes, it poses challenges in understanding its pathophysiology and developing effective treatments. With MODY4 being monogenic in nature, gene therapy using adeno-associated viruses (AAVs) could provide a single-treatment cure. This study aimed to establish a mouse model carrying the most prevalent human point mutation, P33T. Homozygous mice were thoroughly characterized to assess phenotypic manifestations and the suitability of the model for studying MODY4 and the possibility of AAV-mediated gene therapy. We generated a mouse model containing the *Pdx1*^{P33T} mutation using CRISPRCas9 technology and conducted an 18-week phenotyping study examining wild-type and homozygous *Pdx1*^{P33T} mice of both genders (n = 15). Starting at 6 weeks of age, the experimental pipeline included weekly body weight and biweekly blood glucose measurements, intraperitoneal glucose tolerance tests (i.p.GTT) at 8 and 20 weeks of age, and intraperitoneal insulin tolerance tests (i.p.ITT) at 12 and 22 weeks of age. Plasma insulin levels were determined at multiple time points. At the end of the pipeline, pancreatic islets were isolated for glucose-stimulated insulin secretion (GSIS, n = 18) and qRT-PCR analysis (n = 4). No significant differences were observed between wild-type and homozygous *Pdx1*^{P33T} mice. Neither routine blood glucose measurements, nor glucose (i.p.GTT at 20 weeks of age) and insulin response assessments (i.p.ITT at 22 weeks of age) suggested a diabetic phenotype as seen in human patients. Plasma insulin levels at 6, 8, 14, 16, 18, 20, and 24 weeks of age showed no difference between groups. Expression profile analysis and GSIS assay on isolated pancreatic islets supported these findings, as neither *Pdx1* itself nor most of its target genes were misregulated. Our comprehensive phenotypic analysis of a mouse model carrying the homozygous *Pdx1*^{P33T} mutation revealed no significant differences in metabolic parameters compared to wild-type controls. Our work indicates substantial differences between mouse and human PDX1 function in the pancreas.

Further refinement of animal models is necessary to better elucidate the pathophysiology MODY4. We are currently repeating this experiment with a high-fat diet challenge to investigate potential exacerbating factors contributing to MODY4 pathogenesis. At this point, this study may serve as a reminder that findings generated in mice cannot always be easily translated to humans.

P0902

Intravenous administration of AAV9-hARSA improves sulfatide accumulation in the central and peripheral nervous systems in aged mice with metachromatic leukodystrophy

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Metachromatic leukodystrophy (MLD) is an autosomal recessive lipidosis caused by a deficiency of the lysosomal hydrolase, arylsulfatase A (ARSA). Biochemically, the enzymatic defect enables intralysosomal accumulation of the ARSA substrate, galactosylceramide I3-sulfate (sulfatide), a major sphingolipid of myelin in glial cells and neurons. MLD presents predominantly as a central nervous system (CNS) condition with myelin degeneration, however as sulfatide accumulates and the disease progresses, a clear peripheral nervous system disease becomes apparent. Children affected by MLD begin to display progressive neurological symptoms around 2-3 years of age. Symptoms such as ataxia, seizures, and quadriplegia culminate in decerebration and eventual death around 10 years. To date, none of the therapeutic efforts examined address both the central and peripheral manifestations.

Sulfatide accumulation in the ARSA-/- mouse is apparent beyond the age of 6 months (6.1pmol/ug) compared with that of a WT mouse (0.32 pmol/ug), determined by HPLC analysis, prompting our inclusion of only aged mice in our study. Tto demonstrate widespread efficacy, we designed and administered an intravenous (IV) therapeutic, adeno-associated vector serotype 9 using an EF1-alpha promoter that overexpresses ARSA (AAV9-hARSA) in an established (10-12 month old) knock-out mouse model of MLD (ARSA-/-).

Following IV injections of AAV9-hARSA in the aged mouse, cohorts demonstrated reduced sulfatide accumulation in the cerebellum ($p=0.007$), spinal cord ($p=0.00001$) and sciatic nerve ($p=0.03$) at 3 months post-injection. Further in vitro studies using MLD patient fibroblasts demonstrated a similar reduction of sulfatide. Additionally, proinflammatory cytokine measurements of mouse tissue also demonstrated changes in expression following vector administration.

When looking at mouse tissue we observed no immunological effects. In vivo models showed no cytotoxicity following treatment with AAV9. Data presented represents a novel study whereby the viral vector success is based on the efficacy of treating both the CNS and PNS disease pathologies associated with symptomatic MLD.

P0903

Gene therapy for tetrahydrobiopterin deficiency

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6-pyruvoyl-tetrahydropterin synthase (PTPS) is an enzyme responsible for the synthesis of tetrahydrobiopterin (BH_4), and BH_4 in turn is a cofactor for the synthesis of monoamine neurotransmitters and nitric oxide (NO). PTPS deficiency causes severe motor dysfunction and neurodegeneration. Although this disease can be treated by oral levodopa (L-DOPA), the treatment is life-long and symptoms appear shortly after dose missing. In view of the heavy burden of the disease and the improvement in the safety of gene therapy, we plan to develop a gene therapy for PTPS deficiency. First we created mouse models of PTPS deficiency in two ways: (1) Knock in (KI) mice with a Chinese founder *PTS* mutation c.259C>T (P87S) which are likely to have symptoms shortly after birth, and (2) Conditional (inducible) knockout (cKO) mice created by flanking a PTS exon (or exons) with loxP, and after mating the cKO mice with mice expressing tamoxifen inducible Cre (Cre recombinase), symptoms can be induced by tamoxifen in adult mice. Currently, the two strains of mice are both in breeding. The gene therapy expression cassette includes a CMV promoter and three cDNAs (*PTS* + *GCH1* + *SPR*) separated by IRES and 2A peptide. In 293T cells transfected with this tricistronic plasmid pcDNA3.1-BH4, western blot analysis detected the expression of each of the three proteins. AAV9-CMV-BH4 was successfully packaged. Injection of AAV9-CMV-BH4 to the putamen of adult wild-type mice elected the levels of BH_4 and dopamine in the brain. In the near future, AAV9-CMV-BH4 vector will be delivered to PTPS mice by either intracerebroventricular (ICV) injection in newborn mice, and by intracisternal (IC) injection in adult mice. We will evaluate if gene therapy can relieve the symptoms of the mice by rotarod test, clasping test, and open field test. We believe that a safe and effective gene therapy will be important for patients with PTPS deficiency, to improve their life quality and maintain self-esteem, and decrease disease burden.

P0904

The effects of H2S to the biochemical features of aging

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The biological phenomenon of aging raises many scientific questions. Since numerous diseases are based on this process, a thorough examination can provide assistance to masses of people. There have been many theories proposed to explain this phenomenon. Our research is based on the theory of aging caused by mitochondrial dysfunction, specifically focusing on mutations accumulating in mitochondrial DNA. For our investigations, we used the D257A genetically modified mouse model. In this mouse model, a point mutation was induced in the subunit responsible for the proofreading activity of DNA polymerase gamma, resulting in mutations accumulating in mitochondrial DNA. Hydrogen sulfide (H2S) gas transmitter has several positive effects on cellular processes. It is capable of eliminating free radicals and carrying out post-translational modifications on proteins. In our study, we aimed to selectively counteract the aging

phenotype using H2S released specifically in the mitochondria. Within the framework of the treatment, we used the AP39 molecule as a mitochondria-targeted H2S donor. Our hypothesis was that this would induce changes in the signaling pathways of biological processes related to aging. By isolating the protein and mRNA content of the mouse liver, we determined the changes induced by the mutation and our treatment through western blot and qPCR analysis. Primarily, we examined genes and proteins related to oxidative stress, mitochondrial functions, and apoptosis processes. Based on our results, we can state that the mutant mouse model does not accumulate free radicals, and we suspect that the mutation results in a caspase-independent cell death process. Our surprising finding was that the AP219 molecule also had an effect in all cases where AP39 did. However, despite this, we would assert that this effect also proved to be cytoprotective.

P0905

Efficient Co-Delivery of Curcumin and Therapeutic siRNA Using Liposomal Formulation for Osteoarthritis

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Chronic knee and lower back pain due to osteoarthritis (OA) and intervertebral disc (IVD) degeneration (IVDD) have a global prevalence and significantly impact human well-being by impairing mobility. Oxidative stress is a key factor in the pathogenesis of OA and IVDD. One of the promising approaches is the non-viral gene therapy that can safely and precisely restore joints and discs. Our study focuses on developing a cationic liposomal formulation as a non-viral gene delivery tool that could assist in the efficient co-delivery of drugs and biomolecules.

Curcumin is a model drug known to downregulate inflammatory cytokines and free radicals while upregulating collagen and aggrecan, thus reducing pain and aiding regeneration. Luciferase siRNA was utilized for initial screening and prototyping, demonstrating effective transfection into luciferase-expressing chondrocytes (C28/I2 cells).

Preparing liposomes with desirable physicochemical and therapeutic properties is challenging due to the numerous interlinked variables. The QbD approach was used to identify critical factors, screen them, and scientifically optimize them. After initial trials, Failure Modes Effects Analysis (FMEA) was performed to identify critical factors, including concentrations of cationic (DOTAP) and helper lipids (DOPE/DOPC/DPPC), pegylated phospholipids (MPEG-2000-DSPE), and cholesterol. The impact of these critical factors on the responses was studied through a Screening L18 design. The study focused on responses such as particle size (PS), polydispersity index (PDI), zeta potential (ZP), encapsulation efficiency of curcumin (EE), cell viability, siRNA complexation capacity, and luciferase activity. Cell internalization was also determined using fluorescence and confocal microscopy. The responses were fed back into the MODDE software to establish the relationship between the factors and optimize the formulation for the highest cell transfection efficiency.

After optimisation, the cell line and primary chondrocytes were induced with oxidative stress and inflammatory conditions and treated with the optimized liposomes. The effect of curcumin was analyzed through biochemical tests such as Total Antioxidant Capacity, Total Oxidation stress, and Malondialdehyde levels. The optimized liposomes complexed with therapeutic siRNA,

including IL-6 and IL-8 siRNA, were evaluated by qRT-PCR and ELISA on the both the cell line and the inflamed primary chondrocyte cells.

Results indicated that liposomes with DOPE achieved the smallest particle size (<200 nm) and the highest encapsulation efficiency of curcumin and siRNA complexation capacity. Optimum formulations showed high cell viability and gene knockdown efficiency. Confocal microscopy confirmed successful cell internalization of the optimized liposome formulation. These co-loaded liposomes effectively transfected chondrocytes without toxicity and could efficiently reduce the IL-6 and IL-8 levels, and Curcumin efficiently reduced both inflammation and oxidative stress, indicating the potential for further in vitro testing in OA and IVDD models.

In conclusion, this study has provided an optimized formulation for curcumin and therapeutic siRNA co-delivery and established a versatile platform for incorporating other lipophilic drugs and negatively charged genetic materials for various therapeutic applications. The findings hold promise for advancing the treatment of OA and IVDD, potentially improving patient outcomes by addressing underlying pathological mechanisms through targeted non-viral gene therapy.

P0906

Oligonucleotide loaded hybrid nanoparticles for chronic liver inflammation management

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Therapeutic oligonucleotides, such as siRNA, offer significant potential for treating a variety of diseases. However, delivery of naked siRNA faces challenges including non-specific distribution, rapid degradation by endonucleases, and poor cellular uptake. Therefore, developing an appropriate carrier to protect siRNA and ensure site-specific distribution is essential. This project focuses on the preparation and biological evaluation of a fully biodegradable, biocompatible, and macrophage-specific nano-drug delivery system for anti-inflammatory siRNA. Such delivery system may serve as a platform for chronic inflammation therapy. Preparation of a oligonucleotides and cationic lipid (DOTAP) complex by Blight-Dyer technique is initial step for successful oligonucleotide loading. Creation of the complex gives the naturally hydrophilic oligonucleotide molecule overall hydrophobic properties. Consequently, oligonucleotide-loaded nanoparticles based on poly(lactic-co-glycolic) acid were prepared by simple and rapid nanoprecipitation method (NP method). Overall, the ideal formulation after optimization is the one prepared by NP method by PLGA polymer 50/50 E in an amount of 10 mg. The surfactant chosen for the NP method is pluronic 407 in concentration of 0.5%. The resulting properties of the NPs are size around 185 nm, PDI < 0.2 and EE of approximately 45 %. Complexes of 1:3 and 1:2 ratio of negatively charged phosphate groups of oligonucleotide and positive ammonium heads of cationic lipid of DOTAP have not had significant impact on properties of the nanoparticles. Results of the optimized NP method were compared to the widely used double emulsion solvent evaporation (DESE). These methods were evaluated using two oligonucleotides. Encapsulation efficiency, size, PDI and zeta potential have been estimated. The difference between nanoparticles prepared by DESE and NP method were observed also using Cryo-TEM. DESE method leads to formation of two distinct types of nanoparticles, while the NP method provided consistent results in terms of nanoparticles structure and morphology.

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P0907

Evaluation of mRNA delivery to cartilage cells by poly(amidoamine) nanoparticles in models simulating the protein-rich microenvironment of the joints

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Osteoarthritis (OA) is a progressive and degenerative disease of the joints, characterized by the loss and inflammation of the cartilage. Globally, OA affects over 500 million people and poses a major challenge to health systems. Nucleic acid therapies (such as mRNA) have emerged as safe and promising alternatives to stop degeneration and repair the cartilage, with broad use in other applications such as the COVID-19 vaccines. To improve the stability and life span of nucleic acids for *in vivo* use, poly(amidoamine)-based polymeric nanoparticles (PNPs) were developed that stand as a leading alternative to lipid nanoparticles (LNPs) in the delivery of mRNA. These nanoparticles are positively charged and have a cationic core that allows for efficient condensation of nucleic acids. They can also be coated with a PEG-polymer for enhanced stability and electroneutral surface. In this work, we aim to evaluate this delivery system in models simulating or containing synovial fluid and a dense extracellular matrix (ECM) as present in the joints.

Firstly, uncoated and PEG-coated NPs were loaded with GFP mRNA and physically characterized after incubation with a synthetic synovial fluid. While the synovial fluid did not affect the size of PEG-coated NPs (≈50 nm), it did promote a substantial increase in size for uncoated NPs (102 nm in formulation buffer / 735 nm in synovial fluid). Next, we compared the transfection efficiency of these two NP types in a relevant cell line (C28/I2 human chondrocytes), in the presence of DMEM or 10% v/v synovial fluid. The uptake efficiency and GFP expression were analysed after 24 h by FACS, and quantified by the percentage of positive cells and median fluorescence intensity (MFI). All groups showed nearly 100% of cells with NP uptake and ≥80% of GFP-positive cells. The main difference was found in the MFI of uncoated NPs transfected in the presence of synovial fluid, which showed 3-fold lower uptake and 3.5-fold lower GFP expression compared with uncoated NPs in DMEM. The PEG-coated NPs were less affected by the presence of synovial fluid and showed no significant differences in MFI for these two parameters (NP uptake and GFP expression).

Finally, we applied an *ex vivo* model of short-term culture of mouse knee joints. After intra-articular injection of fluorescent PNPs and 24 h culture, frozen sections of the tissue were obtained following fixation and decalcification of the knee joints, and then visualized by confocal microscopy. The uncoated NPs were found in the chondrocytes in the superficial layer of the cartilage, which is a native tissue rich in ECM. This suggests that in the joint, charge interactions between cationic PNPs and the anionic ECM may play a more important role in delivery than the nanoparticle size alone. Further experiments will focus on the efficacy of this system in organ-on-chip models. These polymeric nanoparticles may be a promising alternative for intra-articular delivery of mRNA for therapeutic applications in OA.

P0908

Using IVIS bioluminescent imaging to assess *in vivo* biodistribution of Hermes™ nanoparticles

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IVIS bioluminescent imaging allows for non-invasive, longitudinal, detection of *in vivo* bioluminescent signal. This high sensitivity, high throughput approach enables effective and accurate assessment of live, real time, biodistribution of therapeutics. Evaluating the biodistribution profile of gene and cell therapy products is essential to aid understanding of their pharmacokinetics, efficacy, and safety. Moreover, methodologies for evaluating biodistribution profiles, including *in vivo* imaging, are now listed in the FDA's considerations and guidance for gene and cell therapy products. Additionally, live imaging allows for the reduction of animal numbers, and can remove the need to periodically sample animal tissues for plate-based biodistribution assays. 4basebio designs, manufactures, and supplies application specific synthetic DNA or mRNA. Hermes™, their non-viral delivery system, encapsulates these payloads for vaccine and gene therapy applications. 4basebio's scalable, fully enzymatic linear DNA synthesis process via Trueprime™ amplification technology enables production of synthetic DNA constructs devoid of bacterial backbone or an antibiotic resistance marker. The technology is size and sequence independent, enabling the incorporation of polyA tails >120 bp, and allows for large scale production of linear DNA with improved yields over traditional plasmid DNA fermentation processes. opDNA™ replaces plasmid DNA as a template during the *in vitro* transcription of mRNA, demonstrating higher mRNA yields and avoiding the need for an enzymatic linearisation step. Hermes™ employs both traditional lipids and a cationic ligand to drive payload encapsulation and stability in the liquid state. Using IVIS bioluminescent imaging, we demonstrate the ability to assess the biodistribution profiles of Hermes™ nanoparticles encapsulating mRNA which encodes firefly luciferase, following either intratumoural (IT), intramuscular (IM) or intravenous (IV) administration. For IT, albino C57BL/6 mice were implanted subcutaneously with MC38 cells on the left flank, and subsequently received 0.2mg/kg mRNA upon establishment of tumours. For IM and IV, non-tumour bearing CD-1 mice received 0.4mg/kg mRNA via the hindlimb muscle, or 0.5mg/kg mRNA via the tail vein, respectively. Imaging was carried out at 6, 24 and 48hrs post dose. Upon termination, organs were excised and *ex vivo* imaging conducted, to assess tissue specific bioluminescent signal. Total flux was obtained following quantification of bioluminescent signal using an integrated ROI analysis software. In mice receiving an IT dose, localisation of bioluminescent signal only within the tumour was observed, indicating tumour specific retention of Hermes™ nanoparticles. Similarly, in mice receiving an IM dose, signal localisation was observed in the hindlimb muscle only, again indicating retention within the specific tissue in which nanoparticles were administered. In mice receiving an IV dose, strong signal localisation was observed in the liver, lungs, and spleen. Moreover, specific biodistribution to non-hepatic tissues could be manipulated through modification of Hermes™ lipid and ligand molar ratios. In all animals, the *ex vivo* image correlated with the live images obtained. This highlights the accuracy and sensitivity of IVIS bioluminescent imaging and illustrates the ability to detect therapeutics targeting specific tissues.

P0909

Novel lipid nanoparticles (LNPs) for *ex-vivo* immune cell engineering

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Cell and gene therapy (CGT) is a rapidly emerging medical field that is projected to experience substantial growth in the near future. The effective engineering of immune cells for therapeutic purposes depends on the successful intracellular delivery and expression of nucleic acids. While viral vectors and electroporation are commonly employed for delivering nucleic acids to immune cells, they have several limitations, such as restricted cargo capacity, scalability or deteriorating cell viability.

In contrast, the successful implementation of COVID-19 mRNA vaccines has unveiled the potential of LNP technology in delivering nucleic acids. The non-viral nature and synthetic scalability of lipid materials offer a promising avenue to overcome the aforementioned limitations. Moreover, LNPs are particularly attractive for the delivery of mRNA due to their unique characteristics, including enhanced cellular uptake, endosomal escape, and protection of mRNA from nuclease degradation. However, current LNP products require improvement for effective delivery to immune cells. This is attributed to variations in membrane proteomics and lipidomics of immune cells, which impact LNP transfection. Consequently, there is a unmet needs for immune cell-specific LNPs for nucleic acid delivery.

We have developed an innovative ICT (Immune Cell Transfection)-LNP platform, which features novel ionizable and formulations tailored for effectively delivering nucleic acid to immune cells. Our studies have demonstrated that our proprietary LNPs have exhibited efficient transfection efficiency, surpassing both commercial transfection kits and clinically approved LNP-like formulations. While commercially available LNP transfection reagents designed for immune cells typically require the addition of Apolipoprotein E (ApoE) or Fetal Bovine Serum (FBS) to enhance transfection efficiency, one of our ICT-LNP achieves in average 80% transfection efficiency (% GFP+ cells) in primary T cells under serum-free culturing condition, without the addition of ApoE. Another ICT-LNP formulation can achieve about 70% transfection efficiency (% GFP+ cells) in average in primary NK cells under 5% human A/B serum culturing condition and in the absence of ApoE. Furthermore, when primary NK cells were transfected under serum-free condition, ICT-LNPs performed superior transfection efficiency (>90%). All of these ICT-LNP formulations are well tolerated based on various experimental data, including cell viability, cell proliferation, tumor cell killing ability, and the expression of multiple biomarkers.

These outcomes show that ICT-LNPs can serve as an alternative tool for immune cell modifications, alongside viral vectors and electroporation. This innovation has the potential to accelerate the translation of newly identified targets into clinical practice, providing more options for effective, affordable, and safer treatments for patients.

P0910

Rapid Screening of Exosomal RNA Cargo for Therapeutic Research

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Aim: Exosome RNA therapy is emerging as a pivotal tool in cell and gene therapy, offering several advantages over traditional cell-based methods. Exosomes, as naturally occurring nanocarriers, provide a non-immunogenic, stable, and efficient means of delivering therapeutic nucleic acids. In addition, there are significant advantages in their manufacture, storage and distribution. Exosomes, when engineered through host cell transfection, can effectively deliver therapeutic nucleic acids such as mRNA, miRNA, siRNA, and circRNA. A crucial element in harnessing the full therapeutic potential of exosomes is the development of rapid, efficient methods for their isolation and the characterization of their RNA payload. The aim of this study was to validate a rapid and scalable exosomal miRNA isolation platform for characterizing nucleic acid payloads.

Methods: Fully automated exosomal miRNA isolated from 500 µl of plasma (n=60) was performed using a magnetic bead-based affinity capture system (EXO-NET®, Inoviq Ltd) and a high-throughput miRNA extraction t chemistry (Maxwell® HT miRNA Plasma and Serum, Promega Corporation) on an automated platform (KingFisher APEX, ThermoFisher). Western blot analysis was used to confirm the presence of canonical protein biomarkers. The EV-RNA content was assessed by RNA seq using NovaSeq X Plus system (Illumina) and then targeted miRNAs were validated by RT-PCR.

Results: Exosomal miRNA isolation from 60 plasma samples was performed in less than 2 hours. Western blot analysis confirmed the presence of exosomal protein biomarkers (CD9 and flotillin-1). Small RNA seq analysis identified more than 400 miRNAs in plasma derived exosomes. Further targeted miRNA analysis by RT-PCR showed abundancy and consistent expression of miR191 (Ct: 22.43±1.87), miR-21 (Ct: 21.98±1.96) and miR-186 (Ct: 24.59±2.03) in all plasma samples.

Conclusion: We have established and validated a rapid, scalable and automated platform for exosomal RNA isolation for downstream analysis and characterization. The platform holds great promise for facilitating the development and translation of exosome-based companion diagnostics and therapeutics into routine clinical practice.

P0911

In vivo lipid nanoparticle delivery in an acute myeloid leukaemia mouse model

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Acute myeloid leukaemia (AML) is an aggressive and often fatal malignancy characterised by the uncontrolled proliferation of bone marrow stem cells. Common genetic abnormalities in AML include balanced chromosomal rearrangements, leading to fusion oncogenes that drive the cancer. The fusion oncogenes have a large impact on survival, and there is an unmet need for therapeutic strategies that target the specific oncogenes. CRISPR-Cas9 has demonstrated the potential to disrupt the fusion oncogenes in leukaemic cells and in Ewing sarcoma. However, one of the biggest challenges in gene therapy today is *in vivo* delivery. The usage of lipid nanoparticles (LNPs) is an emerging and promising strategy. In this study, an AML cell line derived xenograft (CDX) mouse model was generated by subcutaneous injection of Kasumi-1 cells, a cell line characterised by t(8;21), in the flanks of three mice. All three mice developed tumours in both flanks. Once the tumours were established, two mice were injected intravenously with LNPs loaded with green fluorescent protein (GFP) mRNA. The third mouse was injected with equal volume of Dulbecco's Phosphate Buffered Saline as a control. The injections were administered in three rounds reaching a cumulative dose of 3 mg/kg. The mice were sacrificed, and the GFP expression was evaluated in kidney, liver, spleen, tumour and bone marrow using flow cytometry. In the bone marrow, GFP-positive cells constituted a measurable quantity (2.11% and 1.79% in treated mice compared to 0.66% in control), with similar results in the kidney (2.26 and 1.88% compared to 0.86% in control). For the tumour samples, only the left tumour of one treated mouse showed an increased number of GFP-positive cells (2.62% compared to 0.49% in control). Despite the liver being the primary organ for LNP uptake, no noteworthy increase in GFP-positive cells was found in the liver; however, there was an increase in median fluorescence intensity in the two mice treated with LNPs (249% and 366% compared to control). There was no difference in GFP signal in the spleen. Our results suggest that gene therapy delivery via LNP injections is feasible and can target both bone marrow cells and tumour-tissue derived from Kasumi-1 cells. This study serves as a proof of principle, indicating the need for further research to optimise the experimental design and method.

P0912

Engineering a functionalized RNA transfer system based on retroviral capsid design

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The existing limitations of contemporary gene therapy approaches primarily revolve around the associated risks of gene insertion by DNA-based systems, such as adenovirus and adeno-associated virus vectors. Given the risks inherent in DNA-based gene therapy methods, the development of mRNA-based gene therapy emerges as a promising and viable alternative. Furthermore, the remarkable success of mRNA vaccines in combating COVID-19 underscores the critical need and growing demand for developing efficient mRNA delivery systems for cellular therapies. Recognizing the infancy of mRNA delivery vectors, we explore the potential of a novel mRNA delivery system developed from an engineered viral capsid protein. These proteins demonstrate the ability to interact with various mRNAs and assemble them into viral-like particles in vitro, offering an alternative to conventional liposome-based methods. The primary goal of our study is to refine this mRNA delivery system for practical application. By modifying the viral capsid proteins, aims to enhance their interaction with mRNAs and improve the assembly process, resulting in efficient and targeted mRNA delivery, particularly for the delivery into neuron cells.

Our experiments characterized the biochemical properties of the engineered viral-like particles, including particle size, the species of binding mRNA, and the mRNA capacity. Modifications allowed surface cross-linking with diverse molecules, including polymers and functional peptides, to expand the system's functional repertoire. The system's ability to transfect mRNA into cell lines and iPSC-differentiated neurons was also evaluated. Challenges in particle purification and optimization were identified, guiding future modifications. Our results showed promising directions for enhancing mRNA delivery and targeting, particularly in neuronal cells. Future work will focus on optimizing assembly conditions and incorporating neurophilic peptides to improve the system's efficiency and specificity for applications. Furthermore, the surface-coating molecules will be changed to shift of tropism among different cells. Our study showed the potential and paves the way for more effective mRNA therapies, minimizing risks associated with current DNA-based methods.

P0913

An Innovative Cationic Lipid Library for Efficient and Tunable mRNA-LNPs

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Lipid nanoparticles (LNP) have demonstrated high efficiency delivering RNA therapeutics in vivo. However, the properties of such nanoparticles obtained with conventional ionizable lipids are often hard to modulate, and especially their biodistribution profile. Such ionizable lipid-based nanoparticles often predominantly end up targeting the liver.

One of the current challenges in the field consists of adjusting the particle chemical composition to the targeted application. Here, we have characterized a library of 10 innovative imidazolium-based cationic lipids as key component of cationic LNPs (cLNP). We disclose their chemical structures and demonstrate their efficacy generating LNPs through characterization of their hydrodynamic diameters, Zeta potentials and encapsulation efficiencies. The resulting particles display high transfection efficiencies and have little to no impact on cell viability in vitro, on HEK293 and CaCo-2 cell lines.

In vivo, the biodistribution of the cLNP highly depends on the cationic lipid chemical structures, targeting mainly lungs and spleen. Among this library, we have identified one cationic lipid as a potent additive in Moderna‘s Spikevax formulation.

P0914

Optimized setup for purifying Extracellular Vesicles from human induced pluripotent stem cells

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Human induced pluripotent stem cells (hiPSC) possess tri-lineage differentiation potential, thus represent a promising cell source for tissue-replacement clinical research. Furthermore, hiPSCs secrete extracellular vesicles (EVs) for long-distance intercellular communication and biomolecule horizontal transfer. Therefore, hiPSC-EVs may act as carriers of nucleic acids or other molecules for gene therapy or regenerative medicine. Yet, the study of EV functionality needs an improved isolation strategy, because commonly used ultracentrifugation-based methods are time- and labor-intensive. In this study, we optimized a scalable and standardizable hiPSC-EV purification platform, testing different filters for clarification, tangential flow filtration (TFF), and sterile filtration. We initially purified EVs using a glass fiber prefilter PreFlow™ UUA to remove cell debris and large contaminant protein. EVs were then diafiltered to remove soluble contaminant proteins and concentrated with a flat sheet membrane under gentle operation conditions. The final EV-solution was sterile-filtered with 0.2 µm Supor™ EKV polyethersulfone membranes. We monitored the process at each step for yield and size distribution by nanoparticle tracking analysis and for purity calculating the particle to protein ratio. The initial clarification step reached 85% yield, while TFF retained 80% of hiPSC-EVs and final sterile filtration reached 78% yield.

P0915

Purifying exosomes to meet manufacturing demand using a gentle, size-based, and scalable purification solution

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A scalable workflow for the purification of exosomes, a type of extracellular vesicle (EV), is a major challenge for therapeutic-grade exosome manufacture. Exosomes are large — between 40 and 150 nanometres (nm) in diameter — and downstream processing includes the removal of much smaller size contaminants such as host cell proteins and DNA. Established technologies for research, R&D, and diagnostic purposes include ultracentrifugation, density gradient separation, gravity separation, or a combination of these methods. Although these technologies generate enriched exosomes suitable for characterization, they can damage the exosome structure, suffer from poor yields and scalability, and require long preparation times, which could reduce biological function. Therefore, these methods are not suitable for manufacturing large quantities of therapeutic exosomes. We describe a workflow for both research and clinical scales of manufacturing. The EV enrichment includes depth filtration to remove cells, Benzonase treatment to degrade DNA/RNA, tangential flow filtration using 750 kDa to concentrate exosomes, followed by either gentle size-exclusion chromatography (SEC) with Cytiva™ superSEC resin or chromatography (MMC) with Capto™ Core 700 resin; the latter combines size exclusion and binding mode for separation different species from the load material. During the primary tangential flow filtration (TFF), a 750 kDa hollow fiber produced 100% recovery of EV while different chromatography steps produced different recovery profiles. For example, superSEC resin showed 7% more EV recovery and 3.3 times faster separation over Sepharose™ CL-2B. superSEC resin demonstrated similar performance at two different scales with three different feed materials. In this presentation, we will demonstrate a start-to-finish exosome production process suitable for clinal scale manufacturing of exosomes harvested from three different cell lines. Both chromatography options mentioned above are amenable to scale-up and packing in large-scale columns for clinical-grade EV manufacturing. Depending on the EV dose requirements, a secondary TFF with 750 kDa is proposed for further concentration of EV before sterile filtration.

P0916

mRNA-based cancer vaccine simultaneously targeting multiple KRAS mutations induces robust anti-tumor immune responses in various KRAS cancers

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Mutations in Kirsten rat sarcoma viral oncogene (KRAS) are prevalent in numerous cancers including pancreatic, colorectal and lung cancers with the mutation rates exceeding 30%. Patients with the KRAS mutation often exhibit poor survival outcomes. Despite numerous efforts to target KRAS mutant cancers with chemical compounds, each compound typically inhibits only the G12C mutation of KRAS. However, we have developed and evaluated an mRNA-based KRAS cancer vaccine capable of targeting a wide range of KRAS mutations with a single mRNA, leveraging our mRNA-based cancer vaccine platform. Our goal is to develop an mRNA-based cancer vaccine targeting multiple KRAS mutations, effectively inhibiting intra- and inter-tumoral heterogeneity of KRAS mutant tumors, which can give rise to therapeutic resistance and recurrence of the disease. We have optimized the mRNA-based cancer vaccine utilizing our established platform to increase the expression and antigenicity of the vaccine. In the LL/2 (murine Lewis lung carcinoma, KRAS G12C mutant) and CT26 (murine colon carcinoma, KRAS G12D mutant) syngeneic models, in vivo efficacy of KRAS cancer vaccine candidates was evaluated, showing a notable reduction in tumor growth. Human cancer cell lines with different KRAS mutations were co-cultured with human peripheral blood mononuclear cells (PBMCs) transfected with our mRNA cancer vaccine to evaluate antigen-specific immune responses against KRAS mutant cancer cells. Cancer cells with KRAS mutations showed significantly decreased cell viability under the co-culture conditions with the PBMCs primed with our mRNA cancer vaccine. Our results indicate that a single mRNA vaccine can target multiple KRAS mutations, thereby enhancing the potential to improve therapeutic outcomes for patients with KRAS mutant cancers.

P0917

Advancing Cancer Immunotherapy: Potent Tumor Growth Inhibition and Enhanced Tumor Immunity via Unmodified mRNA-LNP Vaccines Targeting Dendritic Cells with Ligand-Antigen Fusion

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Messenger RNA (mRNA) vaccines have demonstrated exceptional efficacy in combating the COVID-19 pandemic, sparking the exploitation of this technology to develop vaccines against a

range of infectious diseases and cancers. Cancer immunotherapy represents a transformative strategy that harnesses the immune system's capabilities to target and eradicate cancer cells. In this context, mRNA-LNP vaccines have emerged as versatile and powerful tools to deliver mRNA encoding tumor-associated antigens (TAA) to antigen-presenting cells (APCs), particularly dendritic cells (DCs). This delivery ensures efficient presentation of TAAs on the surface of APCs, leading to cytotoxic CD8+ T lymphocytes activation, which are pivotal in the adaptive immune system.

This work focuses on a specific delivery of anti-cancer mRNA vaccines to DCs. We designed an mRNA coding for a fusion protein composed of a tumoral antigen and a specific ligand (denoted as X) targeting specifically DCs. Upon intramuscular injection (IM), LNP encapsulating mRNA encoding the antigen fused with ligand X will transfect both muscle cells and underlying DCs, enhancing the immune response via (i) direct antigen cross-presentation by transfected DCs and (ii) recapture and cross-presentation by DCs of antigen-X fusion proteins secreted from transfected muscle cells. The proof of concept was demonstrated using mRNA encoding ovalbumin (OVA) as a model antigen and our original LNP formulation based on imidazole lipids, which has demonstrated strong efficacy in delivering mRNA both in vitro and in vivo.

Our findings indicate that IM immunizations of mice with mRNAs made with either unmodified nucleosides (UNR) or modified nucleosides (MNR) encoding OVA and OVA fused with ligand X induced OVA-specific CD8+ T cells both systemically (spleen) and locally (lung). A notable increase of the humoral immune response (IgG) was observed in the serum and in the bronchoalveolar lavage of mice treated with either UNR or MNR OVA fused with ligand X compared to mice receiving either UNR or MNR OVA alone. Using a subcutaneous EG7 tumor model, we observed a significant inhibition of tumor progression and increased survival rates, with ~ 35% of mice achieving complete tumor regression, particularly when the vaccination was done with UNR mRNA coding for OVA-X fusion. Conversely, vaccination with MNR coding for either OVA or OVA-X fusion led to a similar immune response with no impact of X ligand. The antitumor effect is attributed to the induction of an efficient memory immune response resulting from the fusion of antigen with ligand X or the modification of OVA mRNAs. Re-challenge of mice with EG7 cells resulted in complete protection against tumor recurrence for at least three months.

Overall, our data demonstrate effective cellular and humoral responses, underscoring the robust immunogenicity and therapeutic efficacy of antigen-X fusion mRNA-LNP vaccines. These findings provide compelling evidence for the potential of this approach and support further evaluation in clinical trials.

P0918

A straightforward LNP therapies development solution, from microfluidic formulation with Tamara to size and concentration characterization with Videodrop

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Lipid nanoparticles (LNP) therapies development is a challenging task. Using the proper tools and metrics is a key success factor. The solution highlighted here, composed of the VIDEODROP and

TAMARA, enables fast and comprehensive screening and assessment capability for LNP formulation & stability study.

This study takes advantage of the VIDEODROP, a fast & user-friendly nanoparticle analysis instrument, to quantify in a single droplet the size distribution and concentration of LNP samples. The LNPs were synthesized using TAMARA, an easy-to-use microfluidics nanoparticle synthesis platform. It allowed to produce numerous LNP samples varying key formulation parameters such as – microfluidics characteristics: total flow rate (TFR) and flow rate ratio (FRR) – and formulation components: presence or absence of a PEGylated lipid. Size distribution and concentration were measured before and after dialysis for all samples. These metrics were followed, for dialyzed samples, over two months to evaluate and compare their long-term stability when stored at 4°C and at room temperature (RT).

Videodrop evidenced how microfluidics parameters, composition, and dialysis impacted both size distribution and concentration, allowing for the fine-tuning of formulation features. The stability study emphasized the role of PEG to stabilize the formulations over time. LNP samples containing PEG and stored at 4°C keep their size and concentration constant over a month. Without PEG and at RT sizes quickly increase and concentrations drop.

The VIDEODROP and TAMARA are the right set of tools to enable fast, straightforward and comprehensive LNP formulation screening and stability optimization.

P0919

CRISPR RNPs delivery by iron oxide magnetic nanoparticles

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CRISPR/Cas technology has revolutionized genome editing since it provides a versatile toolbox for knockout and knock-in generation, base-editing and transcriptional modulation. Although CRISPR/Cas systems hold great promise in medicine, delivery into target cells is still a matter of concern. Delivery of pre-assembled Cas endonuclease and guide RNA (gRNA) ribonucleoprotein (RNP) complex has emerged as a revolutionary strategy due to its efficacy and safety. Integrating nanotechnology as vehicles for RNPs can also enhance its feasibility *in vivo*. Several nanomaterials can be used, but magnetic nanoparticles (MNP) are particularly attractive because of their safety and tunability. For these reasons, the present work aims to design a nanodelivery strategy through the conjugation of Cas9 or Cas12a RNP with carboxymethyl dextran-coated iron oxide magnetic NP for the gene editing of different cell lines. To address this challenge, we characterized MNP-RNP complexes, obtaining an efficient binding by a combination of both electrostatic and covalent interactions with good stability. Afterwards, we explored the delivery and gene editing efficacy of MNP-Cas12a and MNP-Cas9 in HEK-uGFP and MEF-tdTomato fluorescence-based reporter cells, respectively. We observed that nanocomplexes can be uptaken by mammalian cells, decreasing the number of GFP+ cells and increasing the number of Tomato+ cells. Altogether, these findings indicate that MNP are suitable vehicles for the intracellular delivery of Cas RNPs, which can display specific gene-knockout activity. Genetic disorders are the

main objectives for CRISPR therapeutics, so then we wanted to exploit the potential of MNP-RNP to target and correct the genetic changes underlying such disorders. To this end, we designed specific gRNA to target endogenous genes in genetic human disease cell lines. To sum up, in this study we developed a novel nanotherapeutic strategy for the delivery of Cas RNP into target cells, which exhibit efficient gene editing and is likely to be used as a genome editing tool for the knock-out of disease-causing mutations.

P0920

Maximizing Yield and Functionality in saRNA Production: A Comparative Study of Commercial Kits and In Vitro Transcription Conditions

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Self-amplifying mRNA (saRNA) represents a cutting-edge platform for the generation of vaccines and therapeutics due to its ability to induce robust protein expression with lower doses compared to conventional mRNA. However, saRNA production methods have a profound impact on the cost-effectiveness of the process. Optimizing saRNA production is essential to maximize yield, enhance functionality, and reduce costs.

This study aims to compare different commercial saRNA production kits and *in vitro* transcription (IVT) conditions to identify the most effective protocol for saRNA synthesis. We evaluated four different commercial kits: HiScribe® T7 Quick High Yield RNA Synthesis Kit (New England Biolabs), TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific), RiboMAX™ Large Scale RNA Production Systems (Promega), and HighYield T7 RNA Synthesis Kit (Jena Bioscience). Additionally, we assessed the impact of various factors including nucleotide triphosphate (NTP) concentrations, methods for linearizing DNA templates, amount of DNA template used, as well as incubation temperature and duration during in vitro transcription (IVT). The study focused on synthesizing two different saRNAs encoding reporter genes NanoLuc or GFP, each approximately 8000 - 9000 nucleotides long. SaRNAs were further purified through silica-based columns. RNA yield was quantified through spectrophotometry, while quality was assessed via gel electrophoresis and an RNA integrity assay kit. HEK 293T cells were used for *in vitro* screening of the synthesized saRNAs. Luminescence and fluorescence assays were conducted over time to monitor protein expression levels. Additionally, a cost analysis was performed to evaluate the economic efficiency of each protocol.

Our results showed evident differences between kits in gel electrophoresis profile, RNA integrity and yield of both NanoLuc and GFP saRNAs (10 - 98 µg). Functional assays in HEK 293T cells also demonstrated variations in protein expression between kits. Yield was further improved (up to 170 µg) by optimizing NTP concentrations and IVT temperature and reaction duration. These

optimizations resulted in an up to 50 % reduction in cost per mg of saRNA. The linearization method (restriction enzymes vs PCR) did not significantly impact on yield. Two kits emerged as the most cost-effective under optimized conditions due to their lower reagent costs and higher saRNA production yields and biological activity. Results will be further validated in other human cell lines.

This study highlights the critical importance of optimizing saRNA production protocols to balance yield, functionality, and cost. Our comparative analysis of various commercial kits and IVT conditions offers valuable insights for researchers and manufacturers in the field, aiding in the selection of the most appropriate saRNA synthesis methods based on the specific needs and resource availability. By refining production processes, the practical utility of saRNA can be significantly enhanced, facilitating more efficient and accessible vaccine and therapeutic development.

P0921

Ex vivo CAR-modification of NK cells via mRNA and GMP-compliant scale-up of feeder-free NK cell expansion

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Chimeric antigen receptor (CAR)-modified natural killer (NK) cells represent a promising effector cell type for cancer immunotherapy due to their low risk for adverse effects and their suitability for allogeneic applications. The benefits of allogeneic immunotherapy include the potential for accelerated clinical application through the availability of CAR-NK cells off-the-shelf and the capacity to treat a large number of patients. Consequently, there is a growing interest in the development of Good Manufacturing Practice (GMP)-compliant CAR-NK cell expansion processes. First, this study describes the *ex vivo* modification of human donor peripheral blood mononuclear cell (PBMC)-derived NK cells by non-viral gene transfer in a research-scale approach, comparing mRNA lipid nanoparticles (LNP) and electroporation. Using EGFP mRNA as a reporter, we defined the experimental settings, including pulse code, day of transfection, mRNA concentration, mRNA modification, CAR detection method, and optimal lipid mixture. Applying the reporter mRNA findings to CAR mRNA demonstrates that the molecular structure and length of mRNA have a significant impact on CAR expression in NK cells and are critical parameters for successful generation of functional anti-CD123 CAR-expressing NK cells *in vitro*. Moreover, to obtain a high-quality mRNA-CAR-NK cell product, we optimized the NK cell culture to achieve a high number and functionality of NK cells. Second, scale-up experiments were performed based on the results of our small-scale optimization experiments in which primary NK cells were cultured under different culture conditions. Fresh leukapheresis products from healthy donors were used as starting material and NK cells were isolated by negative selection. Within a cultivation period of 21 days, the NK cells were cultured in G-Rex®100M and G-Rex®500M to investigate the expansion capacity and viability. The biological functionality and phenotype of the cells were evaluated in conjunction with the purity of the culture at harvest after 21 days. We show that the cultivation of highly pure NK cell populations (residual T cells <1.0 %) in G-

Rex®100M or G-Rex®500M was feasible and effective, with an expansion fold-rate of over 200 being reached. The receptor profiles were comparable between NK cells cultivated in small-scale and NK cells from scale-up experiments. The expanded NK cells mediated potent cytotoxic activity against K562 cells in functionality assays. The data provide a platform for the production of clinical-grade, GMP-compliant, feeder-free NK cells in sufficient quantities. Combined with *ex vivo* modification using CAR mRNA, we are paving the way for the development of off-the-shelf CAR-NK cell products and overcoming the challenges in the development of allogeneic cell and gene therapies.

P0922

Harnessing mRNA-based therapy to restore p53 function to suppress growth of p53 mutant cancers

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TP53 is a crucial tumor suppressor gene, and its mutation is a critical driver in a wide variety of cancer types, significantly affecting tumorigenesis and prognosis. Numerous efforts have been made to restore and reactivate wild-type p53 functions for the development of cancer treatment. These efforts include targeting specific mutant forms of p53 with small molecules, preventing p53 degradation by inhibiting its negative regulators and utilizing adenovirus to express wild-type p53. However, many challenges persist due to limited efficacy of these approaches and the diversity of p53 mutations. Herein, we present a more efficient way of restoring p53 functions using in vitro and in vivo cancer models from different cancer types with high p53 mutation frequencies. To achieve efficient restoration of functional p53, we optimized the 5' and 3' untranslated regions (UTRs), the open-reading frame (ORF) and the 5' cap of p53 mRNA. Different cancer cell lines originating from lung, ovarian and pancreatic cancer harboring p53 mutations were employed to evaluate the therapeutic potency of the p53 mRNA. Additionally, the effect of p53 mRNA therapy was examined on normal and p53 wild-type cancer cells to assess its selectivity against p53 mutant cancer cells. We found that our p53 therapeutics can selectively inhibit proliferation of p53 mutant cancer cells by inducing the expression of molecular machinery regulating apoptosis and cell cycle arrest, including PUMA, BTG2 and p21. In vivo efficacy was investigated in xenograft models of lung, ovarian and pancreatic cancer. Reactivation of p53 can synergistically increase sensitivity to conventional anti-cancer drugs and many targeted therapeutics, as evidenced by previous studies. Therefore, our further studies explore the potential of p53 mRNA as a combination partner with conventional or small-molecule therapeutics. Our results show that p53 mRNA therapeutics markedly impeded the growth of xenograft tumors without significantly affecting mouse weights. These results emphasize the potential of p53 mRNA therapy as a novel and efficient approach for targeted cancer treatment, offering significant insights into the selective suppression of p53 mutant cancer cells.

P0923

Efficiency and Dynamics of RNA and CRISPR Delivery with Lipid Nanoparticles to Cells and Tissues

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Over the past decade, RNA therapeutics have made significant strides in treating a variety of diseases, including infectious diseases, certain cancers, and disorders caused by genetic mutations. One of the most notable advancements is CRISPR-Cas9, an RNA-guided programmable gene editing technology with tremendous potential for curing genetic diseases. However, despite the progress in gene editing technology, the effective and specific delivery of CRISPR components to target cells and tissues remains a significant challenge in therapeutic applications.

For delivering mRNA in both *in vitro* and *in vivo* settings, lipid nanoparticles (LNPs) have proven to be a safe and effective technique. They offer several advantages, including high encapsulation efficiency, stability, protection from degradation, and ease of functionalization. Utilizing a ratio of 50:10:39:1 of ionizable lipid, helper lipid (e.g., a phospholipid), cholesterol, and a PEGylated lipid respectively, we achieved homogenous nanoparticle size distributions (100-200 nm) using staggered herringbone microfluidics mixing.

To thoroughly establish and characterize the LNP delivery platform, we assessed its qualitative attributes such as size, encapsulation efficiency, zeta potential, and stability. Additionally, we evaluated its delivery efficiency across *in vitro*, *ex vivo*, and *in vivo* contexts using a fluorescent reporter system with LNPs carrying eGFP mRNA, comparing it to the well-established electroporation method. Our study aims to demonstrate the potential of CRISPR/Cas9 gene editing, focusing on achieving both non-homologous end joining (NHEJ) and homology-directed repair (HDR).

Preliminary results indicated that LNPs effectively delivered eGFP mRNA into various well-established cell lines, primary cells, and even difficult-to-transfect cells, such as human endothelial cells. Promising mRNA delivery was also observed in *ex vivo* mouse brain slice culture and *in vivo* via subretinal injection. Furthermore, efficient NHEJ and HDR gene editing was achieved through LNP-mediated delivery of Cas9 mRNA and synthetic gRNA, with or without a single-stranded oligodeoxynucleotide template, in a reporter cell line. Editing of endogenous targets showed good but variable results depending on the cell type, suggesting areas for further improvement. This comprehensive exploration serves as a foundation for future research in this field.

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P0924

Optimizing *in vitro* transcription methods for enhanced mRNA-based therapeutics

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The rapid development and approval of mRNA-based vaccines for SARS-CoV-2 revolutionized modern medicine. A plethora of human diseases can now be addressed with resources to enzymatic *in vitro* transcription (IVT) for the production of customized mRNA sequences with therapeutic potential. Nonetheless, there is a demand for improved manufacturing processes to synthesize mRNA-based therapeutics with increased yield and quality.

Here, we provide insights into optimized IVT parameters for enhanced RNA-based therapeutics. For this purpose, four different commercially available kits were used for the synthesis of mRNA transcripts, including NanoLuc (879 nt) and NeonGreen (993 nt): i) HiScribe® T7 Quick High Yield RNA Synthesis Kit (New England Biolabs), ii) TranscriptAid T7 High Yield Transcription Kit (Thermo Scientific), iii) RiboMAX™ Large Scale RNA Production Systems (Promega) and iv) HighYield T7 RNA Synthesis Kit (Jena Bioscience). For each kit, different concentrations of nucleoside triphosphates (NTPs) and DNA template, IVT reaction times and temperatures were tested. Following IVT, the resultant RNAs were purified by silica columns and RNA yield was determined by absorbance at 260 nm using a spectrophotometer. RNA quality was assessed through gel electrophoresis and using a RNA integrity assay kit. To address mRNAs functionality, luminescence and fluorescence assays were performed in HEK293T cells upon mRNA transfection. Lastly, we performed an analysis to identify the most cost-effective IVT kits and conditions.

The commercially available kits used in the framework of this study led to the production of mRNAs with very different yields (30 - 105 µg) and quality profiles, which also resulted in different expression levels and stability over time *in vitro*. Furthermore, by optimizing IVT conditions, it was possible to achieve higher yields of the synthesized mRNA transcripts (up to 140 µg), consequently maximizing the cost-effectiveness of the established protocols by 26 %. Considering the higher production yields and improved quality observed by gel electrophoresis, two kits emerged as the most cost-effective for the synthesis of mRNA transcripts.

In conclusion, the refinement of the mRNA manufacturing process is crucial, and ongoing advancements in IVT technology promise to enhance safety, efficacy and accessibility of new mRNA therapeutic modalities.

P0925

Supramolecular Functionalization of PLGA Nanoparticles for Delivery of Gene Editing Machinery

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Gene editing technologies, such as CRISPR/Cas9, have made outstanding achievements in recent years. Such technologies present the ability to modify specific genetic sequences with high precision enabling the correction of genetic disorders, enhancement of cellular functions, and the development of new therapies and disease models. Nevertheless, the efficient and safe delivery of gene-editing components into target cells remains an unmet need. Several advances have been made to develop nanoparticle (NP) delivery-based approaches to enhance the efficiency of gene-editing technologies by protecting the genetic material from degradation, facilitating cellular uptake, and ensuring controlled release at the target site. Polylactic-co-glycolic acid (PLGA) nanoparticles have been extensively studied to be used in gene delivery approaches. PLGA is an FDA-approved biodegradable polymer which can be hydrolyzed and broken down into non-toxic lactic acid and glycolic acid monomers. Polyethylenimine (PEI) is a cationic polymer that can be explored to complex with nucleic acids via electrostatic interactions, thereby protecting nucleic acids from degradation and supporting their intracellular delivery. Taking advantage of PLGA anionic and PEI cationic features, herein we developed nanosized particles via simple supramolecular electrostatic interactions aiming to develop a cost-effective and efficient vehicle for delivery of gene editing machinery to human cells. We were able to develop homogeneous and positively charged PLGA/PEI NPs whose stability is preserved at least by 6 weeks. Such positively charged NPs are able to complex Ribonucleoproteins (RNPs) composed by a GFP-tagged cas9 and a anti-hGFP sgRNA. The complex is formed via supramolecular electrostatic interactions, making this approach a valuable solution to develop vehicles for the delivery of gene editing machinery to human cells.

P0926

Comparative Analysis of Transfection Kits for Linear, Self-Amplifying, and Circular RNA

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From vaccine development to personalised cancer treatment and protein replacement therapies, mRNA technology represents a versatile and powerful tool for several diseases. One of the major challenges of mRNA therapeutics is ensuring an efficient delivery of the mRNA into target cells. The size and secondary structure of the mRNA may affect its transfection rate, translation and stability. In addition to conventional linear mRNA, other RNA types such as self-amplifying RNA (saRNA) and circular RNA (circRNA) also hold significant therapeutic potential. SaRNA is a type of mRNA that encodes a replicase, allowing it to copy the original RNA strand within the cell and amplify its own expression. On the other hand, circRNA forms a covalently closed loop, lacking a 5' cap and a poly A tail, which can provide increased stability and unique regulatory functions.

Therefore, in this work, we aimed to optimise transfection conditions for conventional linear RNA (700 - 1000 bp), self-amplifying RNA (8000 - 9000 bp), and circular RNA (1000 - 2000 bp), encoding NanoLuc or NeonGreen, produced by in vitro transcription (IVT). Five commercially available transfection kits (RmesFect (OZ Biosciences), RiboJuice (Millipore), TransIT (Mirus), JetMESSENGER (Polyplus), Lipofectamine MessengerMAX (Life Technologies)) were tested to deliver RNAs to HEK 293T cells, using different ratios of transfection agent / RNA amount (0.1-0.5 ug) (10 different conditions per RNA type). Transfection efficiency was assessed 20 h after transfection through luminescence and fluorescence assays, while cell viability was evaluated by Alamar blue. For all RNA types, the top ten transfection conditions included RmesFect and Lipofectamine MessengerMAX. These kits yielded luminescence values 2 to 4 fold higher and cell viability above 75%. Jet Messenger demonstrated higher transfection efficiency specifically for linear mRNA, while TransIT was more efficient for cRNA and saRNA. RiboJuice was the least efficient and the most toxic for HEK cells. Moreover, we observed that the three types of IVT-produced RNAs were biologically active, confirming the success of our IVT production pipeline. Results will be further validated in other human cell lines.

In conclusion, valuable insights emerged regarding the suitability of specific transfection kits for different RNA types, which will be pivotal for advancing further applications in the field.

P0927

Novel *in silico* protocol for small molecule discovery enhancing lipid nanoparticle targeting specificity

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In recent years, there has been a significant focus on genomic medicine, particularly on the delivery of mRNA. The primary challenge lies in the unstable nature of mRNA, necessitating a vector to deliver its strand into cells to potentially treat diseases by editing the responsible genes. To ensure that the therapeutic payload reaches the correct target cells, two main types of vectors have been identified: viral and non-viral vectors. This project focuses on non-viral vectors, specifically lipid nanoparticles (LNPs), which could be functionalized by small molecules. These small molecules attached to LNPs, act as binders, targeting specific receptors or tissues, thus enhancing the precision of therapeutic payload delivery. Target specificity is a crucial feature to optimise LNP delivery efficiency and reduce severe adverse reactions, ensuring the therapy is both effective and safe for patients.

To overcome the challenge of delivering mRNA to the correct cells while minimizing risks, a rational pipeline combining physics-based and data-driven approaches has been developed. Initially, a receptor of interest was selected using target discovery methods, ensuring its correlation with the studied pathology (e.g., overexpressed receptors in cancer). Our *in-silico* pipeline is composed of a binding affinity score prediction and a newly developed scoring function able to narrow down billions of small molecules to a few thousand promising candidates. For experimental validation post-*in silico* approach, commercially available compounds from the Enamine database are used. Several datasets were chosen, including the REAL and the Diversity datasets containing 6.75 billion and 48 million compounds, respectively. The Diversity dataset, a subset of the REAL, is especially useful as it comprises structurally diverse compounds that meet biodistribution criteria and represent most of the REAL scaffolds. The Diversity dataset was reconstructed in 3D and screened using a high-throughput virtual screening (HTVS) approach to eliminate unfit candidates failing to bind to the target. The top compounds identified through HTVS are then used to search the REAL chemical space for similar molecules using a similarity approach (such as the Tanimoto score). Discriminating compounds based on their chemical structure similarity helped to retain key features while expanding the chemical space. To select the best compounds from HTVS and the similarity search, a computational approach using multiple algorithms that combined physics-based and AI methods was developed to assess their binding capabilities.

Finally, to approximate real-world conditions, the behaviour of the best-ranked compounds was simulated with its target in a solvation state, mimicking the human body's plasmatic environment. For the top candidates, the impact of a hydrophobic tail was studied to simulate LNP addressing.

In conclusion, this protocol generated a rational-based library of diverse small molecules tailored to enhance LNP targeting specificity by using physics-based and data-driven approaches combined with proprietary algorithms. This paves the way for new solutions in genomic medicine.

P0928

Preventive DNase1l3 gene transfer in a mouse model of pneumonia

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Deoxyribonuclease (DNase) cleaves neutrophil extracellular traps (NETs) and help to ameliorate the inadequate immune response. This has been shown for sepsis and hepatorenal injury. In bacterial pneumonia it is expected that the infectious component is crucial, but as shown for sepsis this does not have to be true. Gene therapy is a rarely used strategy to increase DNase activity. The aim of our study was to prove whether intratrachel non-viral plasmid-based gene therapy using DNase1l3 has a preventive effect in a mouse model of bacterial pneumonia. Plasmid DNA (pcDNA3.0) encoding DNase1l3 was applied intratracheally in a dose of 500 ug. Control mice received saline. After 12 hours pneumonia was induced with S. aureus (10E6 cfu). Bronchoalveolar lavage fluid (BALF) was collected after another 12 hours and used for cultivation, cell counting and extracellular DNA isolation after centrifugation.

All mice survived until the end of the experiment. Bacterial counts were lower in the treated group but the difference was not significant. No significant differences were found in cell counts and extracellular DNA in BALF.

The non-viral gene therapy using DNase 1l3 encoding plasmid did not alter the mouse pneumonia model. It is possible that a combination of DNase 1l3 and DNase 1 are needed to improve the outcome. However, the fact that bacterial counts are not increased suggests that the cleavage of NETs at least does not worsen the infection.

P0929

A comparative analysis of commercial kits for enhanced circular mRNA production: yield, profile, and *in vitro* assessment

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The urgency to tackle COVID-19 pandemic gave rise to a massive breakthrough in messenger RNA (mRNA)-based therapies, a field that has now gained significant attention, despite extensive development and optimization during the past two decades. As a new generation of therapeutic mRNAs, circular mRNAs (circRNAs) may address some of the limitations of linear mRNA, regarding stability and immunogenicity. Therefore, engineering circRNAs has become a new topic of high interest in the field of mRNA and vaccine research. Ubiquitous in eukaryotes, circRNAs are characterized by a covalently closed-loop structure generated through a special type of alternative splicing termed backsplicing. In addition to their protein-coding potential, these molecules are perfect vaccine carriers as they lack the free ends necessary for exonuclease-mediated degradation, granting them extended lifespans as compared to their linear mRNA counterparts.

This study focused on optimizing *in vitro* transcription (IVT) conditions to enhance the yield of full-length circRNA transcripts, while minimizing the production of linear cognates. Four different commercially available kits were used for the synthesis of circRNA transcripts through enzymatic IVT, using T7 RNA Polymerase. These included HighYield T7 RNA Synthesis Kit (Jena Bioscience), RiboMAX™ Large Scale RNA Production System (Promega), HiScribe® T7 High Yield RNA Synthesis Kit (New England Biolabs) and TranscriptAid T7 High Yield Transcription Kit (Thermo Fisher Scientific). The DNA templates encoding for the reporter genes NanoLuc (resulting in a 1435 nt circular transcript) and NeonGreen (1549 nt transcript) under a T7 promoter, were used to assess *in vitro* transcription and translation efficiency.

Multiple variables influencing IVT and circularization efficiencies were explored, namely concentrations of nucleoside triphosphates (NTPs) and cofactors, temperature, time and post-transcriptional strategies to further enrich circRNA species. Following IVT, the resultant RNAs were purified using silica-based columns and total RNA yield was determined by spectrophotometry (Abs260nm). Quality and circularity were analysed through densitometry and gel electrophoresis. Lastly, fluorescence and luminescence readouts were used for functionality

assessment of the produced circRNAs in HEK293T cells. Other types of cells are now under investigation.

Our results showed that the yield for total mRNA produced under manufacturer's recommendations ranged from 53 - 119 µg, while optimized conditions improved the yields up to 30%. Additionally, a 25 - 56% ratio of circular *versus* total mRNA was observed using commercial kit instructions. Importantly, upon protocol optimization, circularization efficiencies were boosted by up to 20%. These results led to the assortment of the optimal kit/condition combinations to achieve high circRNA yields.

In conclusion, our results provide valuable insights to streamline an improved IVT protocol with maximized circRNA production and functionality, crucial for future applications.

P0930

Unlocking the potential of nanoparticle extrahepatic delivery using high-throughput screening to enable expanded machine learning design of novel chemistry

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The promise of nucleic acid-based therapeutics is immense for treating a wide range of diseases. The challenge lies in the ability to deliver these therapies to their intended sites in body, ideally specifically and efficiently. Lipid nanoparticles (LNPs) have emerged as a powerful tool for delivery due to their ability to encapsulate and protect nucleic acids and facilitate their entry into cells. With the approval of ONPATTRO and COVID mRNA vaccines, most development is limited to liver indications and vaccines. Most LNPs accumulate in the liver when administered systemically with no or limited exposure in other tissues and organs.

Liberate Bio is hoping to realize the full potential of nucleic acid medicines by building nanoparticle chemistries using novel cationic ionizable lipids to unlock delivery to extrahepatic tissues. We have developed a high-throughput pooled biodistribution assay focused on non-human primates enabling us to screen as many as 96 formulations simultaneously in a single animal. This has allowed us to generate large amounts of high-quality data in a short timeframe to supply the curated data requirements for improved Machine Learning models.

Biodistribution of the LNP and its cargo is the first step in a pathway to expression and potency of therapeutic nucleic acid therapy. As a subsequent screening step, we have developed an in vivo peptide barcoding strategy that enables sensitive and reproducible measurement of expression in vivo. By labeling mRNA with a nucleic acid barcode, and at the same time labeling the subsequent protein product of that mRNA with a peptide barcode Liberate is able to measure simultaneous biodistribution and expression information of large pools of LNP formulations.

These results include the initial proof of concept data generated using mice with a pool of five LNPs which were independently evaluated individually for expression in several extrahepatic tissues. We show that this method accurately reflects the individual expression of mRNA delivered by LNPs in several extrahepatic tissues, including spleen and lung. These peptide barcodes were recovered utilizing the next generation peptide sequencing platform from Quantum Si.

Liberate has built a robust platform for the screening of novel LNPs in a high throughput pooled format using barcoded mRNA. We have further expanded upon that approach by integrating peptide barcoding into our screening funnel allowing us to rapid screen for biodistribution of mRNA in organs and tissues of interest and then identify those LNPs which also demonstrate productive delivery through robust peptide sequencing. This platform results in much cheaper and quicker screening of LNPs.

P0931

A platform approach for producing engineered extracellular vesicles

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Extracellular Vesicles (EVs), including exosomes, are emerging as promising natural nano-scale platforms for delivering nucleic acids, proteins, and small molecules. However, precision EV engineering has been challenging to date. Such technologies have also been largely inaccessible for a broader community due to technical complexities. Here, we discuss materials and methods to express biomolecules on EVs.

Protein scaffolds were developed and clinically proven to effectively load therapeutic proteins on the surface or lumen of EVs. Chemical linkers were developed to link nucleic acids or small molecules to the EV membrane while preserving their bioactivity and potency. Case studies and clinical data for each EV loading and linking technology will be presented. Technologies described here will be available to Pharma, Biotech and Academic communities under a Research License for broader access. They will also be available to therapeutics companies via contract development and manufacturing services for producing their engineered EVs.

P0932

Aptamers as novel therapeutics for molecular targets in inherited cardiomyopathy

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Phospholamban (PLN) is an essential regulator of Ca2+ transport in cardiomyocytes. It regulates heart contractility through reversible inhibition of the sarcoplasmic reticulum (SR) calcium ATPase (SERCA2a) activity. In systole, unphosphorylated PLN inhibits SERCA2a, preventing Ca2+ uptake into SR, while in diastole PLN gets phosphorylated and opens SERCA2a for Ca2+. Mutation R14del in PLN was shown to lead to increased levels of unphosphorylated PLN in mice and in human patients with aberrant Ca2+ transport. These aberrations lead to the development of

dilated cardiomyopathy, arrhythmogenic cardiomyopathy and consequential heart failure. Symptoms, such as malignant ventricular arrhythmias, usually manifest at the age of 50 and later. However, risks of sudden cardiac death in patients with PLN-R14del increase even in young age. The most widely studied RNA-targeting molecules are ASOs, small interfering RNAs and microRNAs. Aptamers are less studied single-stranded DNA or RNA molecules, which bind a protein by folding into an antibody-like three-dimensional structure. One of the benefits of aptamers is that they can target intracellular, extracellular and cell-surface molecules, while ASOs can be optimized only for intracellular targets.

Aptamers are easy to produce and customize. They efficiently disrupt protein-protein interactions by capturing the target protein. This study aims to test ssDNA and ssRNA aptamers as inhibitors for PLN-SERCA2a interaction, which would facilitate Ca2+ uptake and consequently improve contractility of the myocardium. Aptamers are produced and selected by the SELEX-process. Aptamer sequencing is performed with paired-end iSEQ100 system. Aptamer binding to PLN is assessed by Surface Plasmon Resonance and qPCR. Aptamer binding to PLN *in vitro* is studied on HEK293 and healthy iPSC cardiomyocytes. Inhibition of PLN-SERCA2a by aptamers *in vitro* is studied on iPSC-derived cardiomyocytes with and without PLN-R14del mutation. Ionizable lipid nanoparticles produced by microfluidics are used for aptamer delivery *in vitro*.

Preliminary results show that commercial DNA aptamers in healthy iPSC cardiomyocytes were localized around the nuclei. By 5 rounds of SELEX we managed to produce more than 100 RNA aptamer candidates. After NGS sequencing we chose the aptamers with the highest numbers of reads for the subsequent testing. Initial binding experiments with RNA aptamers produced by SELEX show affinity to PLN *in vitro.* LNP production was optimized for RNA aptamers and the preliminary experiment demonstrated successful delivery of RNA aptamers in HEK293 and iPSC cardiomyocytes. In conclusion, the preliminary results of *in vitro* experiments show that aptamers are attractive novel therapeutic molecules to improve cardiac function in PLN-R14del induced cardiomyopathies.

P0933

Optimization of design and production of a HiExpressTM IVT RNA platform for enhanced transgene expression for IVT RNA therapeutics in vitro and in vivo

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The development of effective in vitro transcribed (IVT) RNA therapeutics necessitates an optimized platform that can enhance transgene expression both in terms of levels and duration. The HiExpress™ IVT RNA platform developed by VectorBuilder integrates a multifaceted approach to optimize RNA sequences and production methods, thereby significantly improving the therapeutic potential of RNA drugs in clinical applications. The platform employs several strategies to achieve optimal transgene expression, focusing on minimizing secondary and tertiary structures that can impede translation, codon optimization tailored to the host strain, and avoiding microRNA targeting sites to reduce unintended gene silencing and degradation. The platform also evaluates various RNA capping methods, UTR combinations, and polyA lengths to stabilize the RNA and enhance translation. Advanced LNP formulations, including tissue-targeting LNPs, are designed to improve delivery and uptake by target cells. Experimental validation of the

HiExpressTM platform has demonstrated significant improvements in transgene expression in both in vitro and in vivo settings, with optimized RNAs exhibiting robust expression and greater therapeutic efficacy. By integrating these comprehensive optimization strategies, the platform addresses multiple facets of RNA design and delivery, resulting in superior performance of IVT RNA therapeutics. VectorBuilder's HiExpressTM platform can be applied to a variety of RNA therapeutics to enhance their clinical efficacy and VectorBuilder offers a full range of CRO and CDMO services for RNA therapeutic development.

P0934

Utilizing CIMmultus OH for efficient purification of MSC EVs

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Mesenchymal stem cell (MSC)-derived extracellular vesicles (EVs) have promising therapeutic potential in diverse applications, such as tissue regeneration and immunomodulation. On the pathway to the clinic, there is a substantial need for developing robust and scalable methods for EV production and purification. In this work, we prepared MSC EVs using two different production systems. 2D production in flasks was compared to a 3D microcarrier-based bioreactor process. Conditioned media EVs harvested from both production strategies were captured using preferential exclusion chromatography on the CIMmultus OH column. This way we concentrated the vesicles and removed the majority of impurities in a single step. Finally, samples from different stages of EV bioprocess were evaluated based on particle yield and the presence of common exosome markers using NTA and PATfix analytics. CIMmultus OH chromatography offers a valuable tool for the EV capture step and demonstrates robust performance on different upstream materials.

P0935

Developing an mRNA nanomedicine platform to democratise therapeutic antibodies

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The manufacturing of therapeutic antibodies requires expensive, complex, and frequently challenging production that keeps the cost of treatment in the clinic high. Alternatively, leveraging novel modalities such as mRNA to encode therapeutics circumvents many of the manufacturing problems associated with biologics, and instead relies on *in vivo* expression of antibodies within patients. Importantly, recent work demonstrates that therapeutic antibodies translated *in vivo* from mRNA in pre-clinical models can be readily detected within hours and often with persistence up to several weeks. Peak levels of circulating mRNA-encoded antibodies

are comparable to recombinant protein equivalents and have been shown to be within favourable therapeutic ranges in first-in-human phase I trials.

Here we outline an mRNA-LNP based platform and roadmap to encode and deliver therapeutic antibodies, such as the standard-of-care anti-HER2 antibody Trastuzumab, using *in vitro* as well as *in vivo* efficacy models for validation. Preliminary *in vitro* characterization of mRNA expressed Trastuzumab shows robust translation in producer cell lines, retention of antigen specificity and heavy chain-light chain integrity, demonstrating proof-of-concept. To evaluate *in vivo* expression of mRNA encoded Trastuzumab, antibody levels were measured in serum after infusing mRNA formulated LNPs in standard pre-clinical and translational pharmacology models. Plasma concentrations of secreted mRNA-encoded trastuzumab were benchmarked against circulating levels of infused recombinant antibody in parallel groups, and pharmacokinetics interrogated. Additionally, bioluminescence imaging was harnessed to understand whole-animal distribution and sites of mRNA-translation when using specific LNP formulations.

Follow up work will focus on understanding the efficacy of mRNA encoded Trastuzumab using *in vitro* cytotoxicity assays (ADCC) with human immune cells and target tumour cell lines. Moreover, *in vivo* anti-tumour activity will be assessed in a mouse tumour xenograft model evaluating parameters such as tumour volume, growth, and morbid-free survival.

Overall, our aim is to leverage a robust platform to demonstrate that mRNA-encoded therapeutic antibodies can provide an effective, alternate strategy for solid tumour immunotherapy and unlock a strategy to democratise and enhance patient access to biologics.

P0936

Property-structure-function analysis of complex LNPs using integrative biophysical, molecular-, and cell-based assays

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The complexity of lipid nanoparticles (LNPs) for delivery of nucleic acids presents a developability challenge. Fit-for-purpose and complementary analytical tools are required to design successful formulations, and to inform robust production of stable, safe, and efficacious products. In this study, different mRNA-LNP formulations and batches were compared using biophysical and cell-based assessments. Fluc mRNA-LNP formulations were compared based on size, particle concentration and zeta-potential with nanoparticle tracking analysis (NTA), dynamic and electrophoretic light scattering (DLS, ELS). Differential scanning calorimetry (DSC) was used along with cell-based transfection efficiency assay to compare higher order structure (HOS) and functional activity of the two batches of Fluc RNA-LNP2 formulation. Differences in physicochemical profiles of Fluc mRNA-LNPs were well-correlated and robustly resolved with DLS, NTA and ESL. Poor HOS comparability of LNP2 batches was corroborated in the functional assay. Taken together, these data suggest that biophysical characterization of LNP vectors combined with cell-based assays to assess LNP transfection efficiency are key for informing association between particle properties, HOS and functional performance.

P0937

Breaching the endosomes: a calcium-responsive phospholipase system for enhanced endosomal escape

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Non-viral gene therapy holds promise for treating a plethora of diverse diseases. However, its advancement still faces many hurdles, including poor endosomal escape. Our goal is to enhance the potency of the non-viral transfection system termed TFAMoplex, which is based on the human mitochondrial transcription factor A (TFAM), a protein used for compacting DNA. The TFAMoplex system also includes a phospholipase C (PLC) for endosomal escape. This study aims to develop a system enabling the recruitment of PLC to the plasma/endosomal membrane and to subsequently release it from the TFAMoplexes in a controlled manner once inside the endosomal compartment. This combined recruitment and release system is expected to enhance endosomal escape and transfection efficiency.

Membrane recruitment of PLC was accomplished by fusing a nanobody binding the ubiquitously present tetraspanin CD9 (CD9nb) to PLC. Trigger-responsive release of PLC inside the endosomal compartment was achieved by employing a calcium-sensitive split protein. Calcium-dependent binding of the two split parts was characterized with a split nano luciferase (nanoLuc) complementation assay. For controlled release of PLC under low calcium concentrations, such as encountered in the endosome, the N-terminal part of the calcium-sensitive split protein was fused to PLC-CD9nb. The C-terminal part was genetically coupled to TFAM, resulting in non-covalent and calcium-sensitive coupling of the two proteins. PLC induced endosomal leakage was assessed by transfecting HeLa cells expressing mRuby-galectin-8, which accumulates in damaged / disrupted endosomes and generates red fluorescence signal. The overall potency of the new system was assessed by transfecting Hela cells with a reporter plasmid and quantified by flow cytometry analysis.

The split nanoLuc complementation assay confirmed the coupling of the two calcium-sensitive split proteins at extracellular calcium concentrations. Binding also occurred in 80% serum. No relevant binding occurred at low µM calcium concentrations, as found in the endosomal system. Enhanced endosomal leakage compared to the control was demonstrated with the Hela-mRuby-Gal8 reporter assay. The new TFAMoplex system containing the calcium-sensitive PLC release and the CD9nb for membrane recruitment resulted in increased transfection efficiency while requiring eight times less PLC compared to the control original TFAMoplex system indicating increased potency.

Membrane targeting and endosomal release of PLC is both simple and effective, making it easily translatable to other protein-based gene delivery platforms.

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P0938

Facilitating the development of mRNA-based drugs: one-stop solution for mRNA synthesis and LNP delivery

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The successful development of mRNA-based COVID-19 vaccines has paved the way for exciting new opportunities in the realm of mRNA-based drugs and vaccines to tackle a range of challenging diseases. Prominent applications include prophylactic and therapeutic vaccines, cell therapy, genome editing, therapeutic antibody production, protein replacement, and more. mRNA-based approaches have many desirable features compared with traditional drug development process, such as easy design and modification, no risk of genomic integration, and rapid adaptation for different diseases by changing the template DNA sequence. However, due to the instability and negatively-charged nature of naked mRNAs, they are unable to directly enter the cells and hard to successfully function in vivo, which means 'delivery' is critical in mRNA drug development. Lipid nanoparticle (LNP) is a highly effective way for mRNA delivery, which has been broadly verified by approved mRNA vaccines and many clinical candidates. Here in GenScript, we provide the full-service solution to IVT mRNA manufacturing that can significantly streamline customers' workflow starting from gene synthesis, plasmid production, mRNA synthesis to LNP formulation. mRNAs and LNPs produced at GenScript are optimized with our proprietary production platform, ensuring the quality and expression efficiency. Through internal testing and collaborations with clients, GenScript has also accumulated extensive experience in mRNA drug development. In one case, we encapsulated the SARS-CoV2 RBD mRNA in LNPs and conducted immunization in mice; the serum test showed that the antibody titers remained at high levels for up to 90 days. In another case, we used mRNA-LNPs for preparation of antibodies against multiple transmembrane proteins; we designed CCR9 mRNA sequence and encapsulated the mRNA into different LNP formulations, and the results showed the mRNA-LNPs could successfully induce corresponding antibody of CCR9 protein in mice. Not only that, we also co-encapsulated the sgRNA and Cas9 mRNA in LNPs for genome editing, and the results showed high gene editing efficiency both in vitro and in vivo. In addition to conventional LNP formulations, GenScript is currently developing some special LNP formulations targeting specific tissues or cells to meet our customers' needs. In summary, Genscript has developed a one-stop solution for mRNA synthesis and LNP delivery, which can effectively facilitate the development of mRNA-based drugs.

P0939

Deoxyribonuclease activity is increased in animal models of systemic, but not local inflammation

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Non-viral gene therapy using naked DNA is a safe alternative to viral gene therapy. The efficiency of gene transfer is often very low due to cleavage of the vector. Deoxyribonuclease (DNase)

activity cleaves extracellular DNA including exogenous naked DNA, but the factors affecting its activity are understudied.

Cecal ligation and puncture was used to induce sepsis as an example of systemic inflammation. Collagen-induced arthritis and dextran sodium sulphate-induced colitis were used as examples of local inflammation. Adult mice were used for all models. DNase activity was assessed using single radial enzyme diffusion gels containing DNA as a substrate.

All models worked - the corresponding scores proved the presence of the relevant symptoms. Sepsis induction caused a 2-3-fold increase in plasma DNase activity ($p<0,001$). Mice with arthritis or colitis had plasma DNase activity comparable to control mice.

Our results show that systemic, but not local inflammation increases plasma DNase activity. This has direct implications for potential non-viral gene therapy targeting these models and/or corresponding diseases. Whether treatment of sepsis requires a higher dose or a protection of the naked DNA should be tested in future research.

P0940

Ionisable lipids from sustainable biorenewable sources for the delivery of RNA-LNP vaccines

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To ensure equitable access to vaccines in lower-middle income countries (LMICs), sustainable manufacturing platforms and reliable access to raw materials are vital. In the case of mRNA vaccines, the lipid nanoparticle (LNP) forms an essential part of the final drug product, as it facilitates cytosolic delivery of the transcript. Traditionally the ionisable lipids used in mRNA-LNP vaccines are derived from petrochemical sources and require multiple purification steps, making them costly to synthesise. Biorenewable sources of novel lipids are therefore attractive, as they promote green product design and sustainable process development pipelines for vaccine manufacturing. We have identified cashew nutshell liquid (CNSL) as an inexpensive biorenewable source of various building blocks for ionisable lipid synthesis, requiring only a single chromatographical purification step. Cashew nutshells are an agricultural waste product that is found in abundance in LMICs such as Cote d'Ivoire, India, Vietnam, Tanzania, and Benin. To date, we have synthesised over 50 novel ionisable lipids from CNSL containing a variety of ionizable or cationic head groups and hydrophobic lipid tails. High quality single- and multiple-component mRNA-LNPs were successfully formulated, characterised, and tested *in vitro* and *in vivo*. The size of the LNPs ranged from 80 to 145 nm (single-component), and 30 to 70 nm (multi-component), with PDIs below 0.3 suggesting a homogenous population. Individual pKa analysis was performed to predict endosomal escape. mRNA was encapsulated with high efficiency (>90%) and show strong reporter gene expression with no significant cellular toxicity when assessed in cell culture. Improvements in delivery efficiency have also been achieved by incorporating plant-based alternatives to cholesterol, such as stigmasterol. *In vivo* biodistribution studies showed CNSL-derived LNP candidates administered by intramuscular or intradermal vaccination achieved localised mRNA expression at the site of injection. No liver toxicity was observed for any of the candidate LNPs (ALT <60 U/L; AST <100 U/L). Amine ionisable headgroups such as

diethanolamines showed improved mRNA expression compared to piperazine headgroups. Shorter hydrophobic lipid chains showed improved delivery compared to longer chains, while inclusion of branched and alkene chains were favoured over alkyne chains. Immunogenicity studies in mice have confirmed induction of antigen-specific cellular and humoral immune responses using CNSL-derived SARS-CoV-2 mRNA vaccines, comparable to an LNP formulation currently used in licenced mRNA vaccines. Mice were vaccinated intramuscularly (prime-boost) following which splenic T-cell activation and serum binding/neutralising antibody responses were measured. Increased secreted cytokine responses (IFN-γ, TNF-α, IL-2, and IL-6) following stimulation with SARS-CoV-2 spike glycoprotein peptide pools confirmed activation of antigen-specific T-cells. Importantly the responses elicited following vaccination with the CNSL-derived LNPs mimicked those obtained with the licenced control. These results support further empirical design of the biorenewable lipid nanoparticle platform for improved vaccine and therapy delivery. In addition, reducing the cost of lipid synthesis while developing green products and sustainable manufacturing will strengthen mRNA-LNP vaccine production in LMICs.

P0941

A new Peptide nanoparticles platform for extrahepatic targeted delivery of therapeutic mRNAs

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Cell and gene therapy medicine for the treatments of various diseases hold a great promises to restore functional version of mutated or missing proteins in patients, yet success in the clinic have been hindered by limitation of current delivery platforms. LNP delivery platform suffer from narrow biodistribution profiles, hepatic accumulation and dosed limiting toxicity. Therefore, current RNA therapeutic applications are limited to local administration, ex vivo gene manipulation or liver-based diseases. To overcomes these issues we have developed an unique RNA therapeutic platform that leverages novel peptide technology able to systemically target and deliver therapeutic RNA into the pathologic cells. Peptide-Nanoparticles technology is based on short amphipathic peptides that form stable nanoparticles with nucleic acids. Peptide/mRNA nanoparticles are stable both in solution and in dry powder formulations, with an average size ranging between 40-100 nm and a high encapsulation efficiency (>90%). Peptide-NPs have been rationally designed for selective organ targeting of functional mRNAs and to overcome hepatic accumulation. We have combined peptide chemistry together with a scalable microfluidic mixing technology to produce nanoparticles with different surface properties making possible systemic multi-application for wide array of extrahepatic tissues.

The potency of Peptide-NPs to deliver mRNA specifically in the lung, spleen, muscle and tumors has been validated in several animal models. We demonstrated that intravenous administration of ADGN/mRNA encoding for luciferase nanoparticles (0.5mg/kg) resulted in mRNA accumulation in targeted tissues with protein expression starting 6 hrs after administration and an optimal expression after 24 hrs.

Systemic administration of Lung targeting Pep-NPs mediated effective mRNA delivery in the deep lung region, mainly in pneumocytes type 1 and type2 epithelial cells. To asses therapeutic gene editing the efficacy, Pep-TL-NPs containing mRNA-CAS9 and sgRNA targeting luciferase, was evaluated for gene editing on orthotopic lung ($H358^{Luc+}$) cancer model. Intravenous administration of Pep-NPs/mRNA (0.5mg/kg) leads to CAS9 expression mainly in the lung and totally abolishes

luciferase expression in the tumor resulting in 90% luciferase gene editing in the lung tumor. No Cas-9 expression was detected in the liver.

Peptide-NPs Tumor targeting have been successfully used for the rescue of lost function of p53 tumor suppressor functions as a potential therapeutic approach in cancers. P53 mutations resulting loss of function are common across variety of tumors and influence tumor cell proliferation and metastasis in more than 50% of cancers. ADGN-531 combines a P53 derived mRNA with a tumor selective Pep-NPs. Systemic IV-administration of ADGN-531 resulted in dose responsive tumor growth inhibition in SaOs2 (osteosarcoma), SW403 (colorectal cancer) and H358 (lung cancer) xenografts. ADGN-531 (3.0mg/kg) prevented tumor growth and resulted in strong tumor regressions.

Pep-mRNA-NPs treatments are well tolerated, no sign of inflammatory response, liver or kidney toxicity or adaptive immune response was detected after single or repeated administrations.

ADGN nanoparticles constitute a safe and effective platform for tissue-specific delivery of functional mRNAs with potential clinical applications. Our results demonstrated the feasibility of extrahepatic mRNA delivery achieved by fine tuning the sequences and biophysical property of Pep-nanoparticles as promising platform for advancing clinical development of mRNA based genetic medicines.

P0942

Determining the effect of microRNA-379 on selected hallmarks of cancer in an immune competent model of metastatic breast cancer

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MicroRNA-379 (miR-379) has been hailed as a potent tumour suppressor, with our group and others generating compelling *In Vitro*, *In Vivo* and clinical data supporting potential for therapeutic targeting in the breast cancer setting. However, studies employed immunocompromised animal models and did not examine the impact of the host immune response. This study aimed to further investigate the role of miR-379 and its impact on cancer hallmarks in an immune competent model of metastatic breast cancer. 4T1 murine breast cancer cells stably expressing luciferase (4T1) were transduced with miR-379 (4T1-379). 4T1 (n=8) or 4T1-379 (n=8) cells were injected orthotopically into the mammary fat pad of immune competent mice with a further n=8 control mice receiving no cells. Weekly IVIS imaging was employed to monitor disease progression. Following study endpoint, tumours, lungs and bones were removed and fixed in RNAlater or 4% paraformaldehyde for proteomics or immunohistochemistry (IHC) respectively. Mass spectrometry was carried out to determine differentially regulated proteins with functional enrichment analysis performed to identify pathways implicated by miR-379 enrichment. Bone remodelling was investigated by Haematoxylin and Eosin (H+E) and MOVAT staining. Selected cancer hallmarks including proliferation (Ki67), angiogenesis (CD31, LYVE1) and immune response

(CD45, CD80, CD206) were examined in tumour tissue by IHC. IVIS Imaging revealed rapidly developing tumours with an apparent reduction in tumour growth in mice with miR-379 enriched tumours. This was reinforced *Ex Vivo* with a significant reduction in tumour volume (P=0.05) and Ki67 expression (P=0.01) in the 4T1-379 tumours compared to control tumours. No significant change in expression of CD31 or LYVE1 was observed. Proteomic analysis revealed a potential role for miR-379 in multiple pathways critical to tumorigenesis including immunity and bone remodelling. Furthermore, proteins associated with alternative M2 macrophage activation (Arginase-1 and Siglec1) were found to be significantly downregulated in miR-379 enriched tumours. Preliminary analysis using IHC reinforced this finding with an apparent increase in M1 macrophage associated CD80 and corresponding decrease in M2 macrophage associated CD206. This highlights a potential role for this miR in regulation of macrophage polarisation. Moreover, significant remodelling of bone was evident in tumour bearing animals compared to healthy controls upon H+E staining. Interestingly, femurs from mice bearing miR-379 elevated tumours appeared to retain their morphology similar to that of healthy controls with maintenance of an intact growth plate and reduced loss of trabecular bone. This suggested miR-379 may play a role in the reduction of bone remodelling which is critical for the development of distant metastasis. The promising data presented highlights a potential role for miR-379 in the regulation of tumour growth and bone remodelling, which may have important implications in disease progression and response to therapy.

P0943

Development of a non-viral genetic medicine for X-linked Alport syndrome by targeted transcutaneous ultrasound-mediated gene delivery

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X-linked Alport syndrome is a prevalent genetic condition characterized by kidney disease, as well as hearing loss and eye abnormalities. Kidney disease in Alport syndrome is caused by a dysfunction of the glomerular basement membrane (GBM) due to a monogenic mutation in the COL4A5 gene encoding the type IV collagen a5 chain suggesting gene replacement therapy as a treatment option for Alport syndrome. However, the lack of safe and effective renal gene delivery approaches has prevented the development of genetic medicines for Alport syndrome. Transcutaneous ultrasound-mediated gene delivery (UMGD) offers a noninvasive and targeted gene delivery approach that overcomes existing delivery challenges.

To assess the potential of UMGD for safe and effective gene delivery to the kidney, SonoThera has developed a technology platform that employs proprietary acoustic energy profiles and commercial ultrasound components for the noninvasive and targeted delivery of next-generation nucleic acid payloads. *In vivo* bioluminescence imaging of firefly luciferase reporter gene expression confirmed efficient gene delivery to normal as well as Alport syndrome mouse model kidneys in a rapid, durable, redosable, and titratable manner. Durable transgene expression was observed for over one year following a single UMGD treatment. Utilizing RNAscope and snRNA-seq analysis, biodistribution of transgene delivery was evaluated in mouse and non-human primate kidneys confirming widespread transgene expression in multiple kidney cells including clinically relevant cell types like podocytes, tubular epithelial and endothelial cells. Safety

assessments, including clinical observations, blood urea nitrogen, creatinine levels, and a proinflammatory cytokine panel, indicated excellent tolerability and safety following single and repeat treatments in mice and nonhuman primates.

Multiple codon-optimized COL4A5 open-reading frame sequences under the control of podocyte-specific promoters were engineered and screened in vitro in primary human kidney cells with the goal of developing a podocyte-specific Alport syndrome gene therapy vector. The top performing COL4a5 vector candidate was tested in vivo utilizing a UMGD targeting the kidney of an Alport syndrome mouse model confirming a high level of COL4a5 expression.

Efficient COL4a5 gene therapy payload delivery to podocytes of the Alport syndrome mouse model kidney along with the favorable safety profile of noninvasive targeted UMGD delivery supports translation of this approach towards clinical development for the treatment of the Alport syndrome.

P0944

vMiX™, an innovative AAV-based RNA interference platform: from *in vitro* development to *in vivo* validation by targeting genes involved in ALS

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Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disorder due to the degeneration of motor neurons in the brain and spinal cord. It causes a progressive loss of voluntary muscle control, resulting in paralysis and fatal respiratory failure. Mutations in several genes have been identified in disease pathogenesis, but the mis-accumulation of TDP-43 drives disease pathogenesis in ~95% of patients. Expanded polyglutamine repeats in the Ataxin-2 gene (*ATXN2)* are a risk factor for ALS and Frontotemporal Dementia. Moreover, knockout and knockdown of ATXN2 in mice significantly slows disease progression in TDP-43 overexpressing mouse models, thus, silencing a gene like *ATXN2* has emerged as a therapeutic strategy to treat ALS.

To enable RNA interference, we generated a novel adeno-associated viral vector expressing a transcript encoding micro-RNA (miRNA) that can rapidly be modified to generate guide sequences that target any transcript in any species. miRNA processing involves various enzymes that sequentially cleave primary miRNA (pri-miRNA) transcripts to generate guides within the RNA-induced silencing complex capable of binding to specific mRNA sequences leading to their degradation of suppression of translation. Our vector has a hybrid miRNA scaffold designed to optimise miRNA processing by Drosha and Dicer and enhance guide strand selection. Moreover, the pri-miRNA expression employs pre-mRNA 3' end cleavage in its processing, demonstrating for the first time that an RNA of interest can be expressed outside the canonical [promoter_transcript_termination-signal] design.

The performance of our vMiX vector was compared to miR-155 miRNA constructs with identical guides and assessed using luciferase reporters. The miR-155 vector exhibited variable levels of knockdown (30-75%) depending on the targeted mRNA region (5'UTR, coding sequence, 3'UTR), while our vMiX vector, expressing the same miRNAs consistently achieved an 80% knockdown.

To test the specificity of our new vector, the knockdown was evaluated against a wild-type and a codon-optimized mRNA coding for the same protein (3 mutations outside the seed sequence). Using protein analysis, vMiX showed an 81% knockdown of the wild-type target but had no activity against the codon-optimized one, demonstrating that, unlike endogenous miRNA, vMiX™ requires a full sequence match to induce silencing, thereby increasing specificity and reducing the risk of off-target effects.

vMiX™ was developed as an RNAi platform. Using *in silico* design we generated multiple miRNA candidates targeting genes implicated in ALS pathogenesis including superoxide dismutase 1, *C9orf72*, and *ATXN2* and assessed their potency for knockdown and processing by quantifying guides and guide:passenger ratio across multiple species (human, primate, mouse, and pig). Two separate *in vivo* studies were carried out using miRNA targeting human *ATXN2* in a mouse model expressing the human transgene with 72 CAG repeats. Intrathalamic injections of an AAV9-miR-ATXN2 in adult mice revealed a dose-dependent response in *ATXN2* mRNA knockdown.

vMiX™ demonstrated highly specific and robust *in vitro* and *in vivo* knockdown, and efficient targeting in multiple cell types. While the vMiX vector designed to target *ATXN2* holds promise for treating sporadic ALS its potential extends to silencing other targets of interest for addressing various disorders in brain and other organs.

P0945

Exploring mRNA lipid nanoparticles for ex vivo T cell engineering in cancer immunotherapy

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Chimeric antigen receptor (CAR) T cell therapy is a promising strategy in cancer immunotherapy. T cells are isolated from the patient and subsequently engineered to express CARs, which are able to recognize and destroy tumor cells specifically. However, current CAR T cell engineering is based on stable transduction with viral vectors. This leads to a permanent CAR expression, which can give rise to several concerns including safety issues and exhaustion of CAR T cells. Therefore, we aim to use lipid nanoparticles (LNPs) as a nonviral method to deliver larger mRNA molecules (encoding for CAR or CRISPR Cas9 nucleases) into T cells. Hereby, CAR or Cas9 can be expressed transiently, decreasing the risk for adverse reactions. Moreover, mRNA-CAR T cells could allow for repeated dosing of highly functional CAR-T cells with less risk for exhaustion. Here, we investigated the influence of LNP charge and the cell culture medium composition on the transfection efficiency. Moreover, mRNA kinetics were evaluated in order to achieve potent CAR T cell therapies. Additionally, the use of suitable LNP delivery vehicles for generation of mRNA-CAR T cells, was determined.

First, various LNPs, encapsulating eGFP encoding mRNA, were formulated including the ionizable C12-200 and cationic DOTAP lipids through microfluidic mixing. Next, activated primary human T cells were transfected with neutral or positively charged LNPs in serum-free media substituted with selected targeting proteins (*e.g.*, ApoE, transferrin). These specific proteins are hypothesized to bind on the surface of the LNPs as a function of their composition. This would enable for the LNPs to bind to specific receptors on T cells and hereby improve T cell internalization and transfection efficiency. Flow cytometry results showed high transfection efficiency and low cytotoxicity for the ionizable LNP in the presence of ApoE. Moreover, an increase in transfection

rates was observed depending on the activation status of the cells. On the other hand, the cationic LNPs transfected T cells independent of targeting proteins and activation status, indicating a distinct transfection mechanism. Nevertheless, moderate cytotoxicity was present, making optimization of the cationic LNPs preferable. Subsequently, we evaluated mRNA kinetics in order to achieve potent CAR T cells. Results showed an extended mRNA expression (encoding for eGFP) for over more than 96h, depending on the presence of additional ApoE. These data support previous findings, namely the presence of extracellular available proteins (*i.e.*, ApoE) can provide enhanced transfection rates for the ionizable C12-200 LNP. At last, this formulation was evaluated for delivery of anti-CD20 CAR encoding mRNA and further experiments will be conducted to confirm the suitability of LNPs for delivery of larger mRNA cargo (*e.g.*, Cas9 mRNA).

In conclusion, our results demonstrate that the combination of LNP charge, extracellularly available proteins, and activation status has a profound impact on the transfectability of T cells by LNPs. Moreover, LNP-based transfection allows for sufficient delivery of mRNA, leading to a sustained protein expression. Ongoing research is focused on further evaluating mRNA-induced CAR expression and subsequent tumor cell killing efficiency.

P0946

Precision immunomodulation of allogeneic T cells *ex vivo* using siRNAs

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The field of cell-based therapies is expanding, opening new horizons for treating cancerous diseases such as lymphoma and leukemia, as well as autoimmune disorders like rheumatoid arthritis and multiple sclerosis. Allogeneic T lymphocytes can eradicate malignant cells through the Graft-versus-Leukemia effect (GvL), but they can also trigger Graft-versus-Host disease (GvHD). GvHD is a severe and life-threatening condition that occurs in up to 50% of patients after hematopoietic stem cell transplantation. Conventional GvHD drugs are non-selective, failing to differentiate between GvL and GvH effects, resulting in high toxicities and relapses. Our vision is to use siRNAs to pre-treat cell therapies such as donor lymphocyte infusions to selectively modify the phenotype of allogeneic T cells and thus prevent development of GvHD while enhancing GvL.

We designed fully chemically modified and cholesterol-conjugated siRNAs targeting 4 genes that were upregulated in GvHD animal models (AURKA, RAN, WAPAL, KIF15). We selected leads with superior silencing potency and tested these siRNAs in an *in vitro* GvH model. siRNA targeting *RAN* showed a potent and dose-dependent T cell inhibition (up to 83%). Silencing *AURKA*, *WAPAL* and *KIF15* alone was insufficient to support T cell inhibition in GvHD model. However, RAN-siRNA also inhibited bead-activated T cell proliferation indicating lack of selectivity to GvH. Formulation of WAPAL-siRNA into extracellular vesicles achieved up to 50% enhancement of T cell inhibition, selective to GvH setting.

Combining siRNAs targeting different genes enhanced T cell inhibition. We identified a potent siRNA mix consisting of AURKA, RAN and WAPAL siRNAs. This siRNA combination inhibited allogeneic T cells up to 90% without compromising curative GvL effect *in vitro*. Surprisingly, the effect of 3 siRNAs combined surpassed the inhibitory effect of single siRNAs, indicating synergistic biological functions of these genes in GvHD. The exchange of 1 siRNA of the 3 diminished inhibitory effect up to 2-fold. The lead siRNA mix is currently being tested in an *in*

vivo study with humanized mice. To understand the synergistic interactions and pathways influenced by our siRNAs targeting *AURKA*, *RAN* and *WAPAL*, we analyze differential gene expression of allogeneic T cells after siRNA mix treatment *via* RNA sequencing. To elucidate the mechanism underlying the potent siRNA mix, we are phenotyping different donor-recipient pairs of T cells by analyzing T cell differentiation after siRNA treatment *via* T cell subtype-specific antibodies and secreted cytokines using flow cytometry. siRNA treatment shifts the T cell phenotype towards regulatory T cells with variability on the individual donor-recipient pair.

Given the rapid division of T cells and the dilution of siRNA concentration with each division, we analyzed the silencing activity over time in bead-activated T cells. Silencing *RAN* gene with a 4 µM single siRNA inhibited cell growth and induced cell death, whereas 4 µM single siRNAs targeting *AURKA*, *WAPAL* and *KIF15* maintained 50% of silencing up to 12 divisions post-treatment.

Our data provide proof-of-principle for siRNA mixes as a precision immunomodulatory therapy for GvHD and can be envisioned as a clinical concept for *ex vivo* treatment of stem cell grafts and /or donor lymphocytes.

P0948

Why advanced, orthogonal analytics is crucial in LNP development – and how we might get rid of TFF

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Development and use of mRNA vaccines and therapeutics is crucially dependent on safe, efficient and manufacturable delivery vectors. Beyond viral vectors, lipid nanoparticles (LNPs) have emerged as the lead delivery platforms, convincingly demonstrated in the Covid-19 mRNA vaccines and in a wide range of ongoing clinical trials.

Nanoparticle-based delivery vectors, even fully synthetic like LNPs, are complex systems that have very distinct requirements in terms of analytics and characterization, both for rational development, during scale-up and in clinical manufacturing. Analytical methods for small molecules, peptides or protein drugs are frequently *not suited* for nanoparticles, and novel drug attributes are considered critical, like particle size and size distribution.

In the current work, the *selection, suitability and application of analytical methods* – and their orthogonality and complementarity, as requested by regulatory authorities – is presented, based on comprehensive comparisons and interlaboratory studies we have performed. A tiered analytical approach is proposed, from the early screening by batch methods like dynamic light scattering (DLS) or nanoparticle tracking analysis (NTA) to more in-depth analyses with e.g. multi-detector field flow fractionation (MF-FFF), differential scanning calorimetry (DSC) and analytical ultracentrifugation (AUC). We show that the latter methods are crucial to discover more subtle differences in LNP quality that are important for biological activity. The drug substance – mRNA – is an equally challenging analyte, and methods based on e.g. capillary gel electrophoresis (CGE) and mass spectrometry (LC-MS/MS) are useful.

Finally, a novel separation technique for *intact LNPs* based on their surface chemistry is presented. Beyond the new analytical opportunities, this could also hold significant potential to

facilitate purification and buffer exchange on a large preparative scale, alleviating challenges inherent to the current dialysis and tangential flow filtration (TFF) methods used extensively in both academia and industry.

P0950

Precision miRNA therapies: Fully modified miRNA mimics to treat Graft vs Host disease while maintaining Graft vs Leukemia

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Graft versus Host Disease (GvHD) remains a significant complication following allogeneic hematopoietic stem cell transplantation, occurring in up to 50% of cases and resulting in a survival rate of less than 50% within seven months post-acute GvHD. Current GvHD therapies often induce immunosuppression, which undermines the immune system's ability to eliminate residual leukemic cells - Graft versus Leukemia (GvL) effect -, leading to high relapse rates. Different miRNAs have been reported to be downregulated during GvHD but preserved during GvL, presenting a potential target for therapy.

We screened a panel of chemically modified cholesterol-conjugated miRNA mimics in order to identify a clinically relevant chemical scaffold for GvHD therapy. For that, we tested the impact of the metabolic stability of the antisense strand as well as length and degree of complementarity of the sense strand of miR-146a, miR-374b and miR-181a mimics. These three miRNAs have been shown to be down-regulated during GvHD but that their expression does not compromise GvL.

To assess miRNA mimic silencing, we cloned complementary target sites for each miRNA into a dual luciferase reporter plasmid and transfected HeLa cells. We tested efficiency on natural targets using probes against *IRAK1*, *AKT1*, and *PTPN11* for miR-146a, miR-374b, and miR-181a, respectively, and quantified by QuantiGene™ assay. As a functional assay, we performed mixed lymphocyte reactions (MLR) to evaluate miRNA mimic efficacy in a GvHD model, tracking T cell proliferation with cell division dyes. We modified the MLR by adding MOLM-13 cells to create a GvL model, ensuring miRNA mimics did not impair T-cell-mediated anti-leukemia effect.

Fully matched sense strands impair miRNA mimic silencing efficacy on completely complementary reporter targets up to 6-fold and abolish efficacy on validated natural targets compared to natural mismatched sense strands. These trends hold true in the context of all three miRNA sequences tested and are enhanced with shorter lengths of sense strands. Higher phosphorothioate content is associated to two times with higher silencing potency compared to lower phosphorothioate content. In the functional assay of GvHD, fully matched sense strands were superior (2.3-fold improvement) to mismatched natural sense strands unlike in silencing assays. General effects of chemical modifications on the miRNA targetome may explain this observation. Higher phosphorothioate content associate with more potent GvHD inhibition (up to 4-fold improvement), similar to silencing assays. Surprisingly, different chemical structure of the same miRNA sequence with very similar efficacy in GVHD models showed differential impact in GvL models, with structure of higher phosphorothioate content tending to compromise GvL (up to 10-fold increase in leukemia growth), especially in the context of miR-374b. RNA-Seq data reveal targetome differences between these miRNA mimic versions.

Our findings demonstrate that both sequence and chemical modifications synergistically contribute to the physiological efficacy of miRNA mimics, affecting differently the targetome and defining GvHD specific mechanisms. Fully modified miRNA mimics emerge as promising candidates for GvHD therapy, offering a potential strategy to mitigate this severe post-transplantation complication while preserving the critical GvL effect.

P0951

A platform for multiplexed cell engineering - silicon membranes for robust mechanical delivery of diverse cargos to human stem and immune cells

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Cell therapies with engineered immune or stem cells have the potential to revolutionize many areas of unmet clinical need including cancer, infectious disease, autoimmunity and even neurological diseases. RNA-based and genomic editing technologies (e.g. CRISPR/Cas9) have immense potential to enable design of cellular therapies with customized functionalities. One of the major challenges, however, is the difficulties of delivering these cargos into the desired cell types. Technologies like electroporation and viral transduction have underpinned the majority of first generation cell therapies yet have struggled to enable the generation of more sophisticated and cost-effective next generation therapeutics. Issues with cargo limitations, non-specific disruption of cell phenotype, and cost have been a major impediment to progress.

At Portal, we have developed a silicon membrane-based delivery technology that dramatically simplifies immune and stem cell engineering by mechanoporation. Our results in primary T cells, for example, have demonstrated the ability to deliver a CRISPR RNP and mRNA simultaneously with efficiencies over 80% while maintaining over 70% viability. Moreover, our work has demonstrated conservation of critical functions within the cells, such as the ability to proliferate rapidly upon stimulation post-delivery. The Portal platform has already been successfully implemented for a variety of other cell types including NK cells, monocytes, B cells, HSCs and iPSCs. The simplicity of the delivery strategy also facilitates cost-effective implementation at scale whereby silicon membranes close to 1 cm^2 in size are capable of treating over a billion cells by simply connecting to existing GMP equipment as an in-line consumable.

Our technology is currently being implemented for research and clinical scale use, including point-of-care manufacturing. With the ability to deliver many cargos simultaneously to a variety of cell types in a simple format, using both RNA-based and genomic editing technologies, we aim to unlock vast biological potential in the field of stem and immune cell therapy. Coupled with reduced cost and compatibility with a variety of GMP infrastructure, one could potentially have a substantial impact on the number and diversity of patients served with next generation cellular therapeutics.

P0952

Intracellular PLGA-nanoparticle-mediated delivery of adenine base editor RNP complexes to target sickle cell disease in erythroid cells

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Ex vivo gene editing of $CD34^+$ haematopoietic stem and progenitor cells (HSPCs) holds significant promise in treating various genetic diseases, including sickle cell disease (SCD) and other haemoglobinopathies. While a number of genome-editing-based clinical trials are ongoing, with the first one approved in 2023, they mainly rely on electroporation and viral-based delivery of the CRISPR complex, often associated with cytotoxicity. Nanoparticle (NP)-based genome editing approaches can potentially enhance the gene-editing capabilities of HSPCs and minimise the cytotoxicity of the treatment, while offering a viable *in vivo* delivery system. SCD is caused by a single nucleotide substitution in the β-globin gene (*HBB*) leading to the production of the HBB^S globin chain. Induction of foetal haemoglobin (HbF) in high enough levels or changing of the HBB^S into the Makassar variant HBB^G can rescue the disease phenotype. The aim of this study was to develop an *in vivo* therapeutic-agent delivery system to treat SCD through two different correction pathways using adenine base editing (ABE). In a previous study, CRISPR/Cas9-PLGA-NPs were developed, that effectively encapsulated Cas9 protein and a single guide RNA (sgRNA) that facilitated gene editing at the γ-globin gene locus, leading to increased HbF expression in primary erythroid cells. Human HSPCs internalised and processed CRISPR/Cas9-PLGA-NPs without experiencing cellular cytotoxicity. In this study, in-house produced and purified ABE8e with NGG and NRCH PAM recognition sites were used to target the *HBG* promoter and the *HBB* gene in SCD-derived patient lines to increase HbF expression or change the SCD mutation into the Makassar variant, respectively. We assessed the editing efficiency of different RNP complexes and the subsequent downstream effects on the globin protein level, through western blots, flow cytometry and HPLC. Sickling assays showed that some of ABE treatments led to reduced sickling of the cells. These combinations were subsequently effectively encapsulated in the PLGA-NPs, carrying the ABE8e of interest, sgRNA and a fluorescent probe. The SCD-derived patient lines were used for a comparison of the editing efficiency and cytotoxicity between the two delivery methods. This advancement in CRISPR/Cas9-PLGA-NP technology provides a promising tool for delivering CRISPR components to target HSPCs. This way we may overcome the limitations and risks associated with both *ex vivo* gene therapies relying on haematopoietic stem cell transplantation and *in vivo* viral-based therapies and potentially paving the way for long-term-therapeutic *in* vivo treatment of β-haemoglobinopathies and other genetic disorders.

P0953

FlashRNA®: a game-changing RNA therapy for regenerative medicine

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Current gene & cell therapy approaches show that there is no universal delivery technology for all strategies. As DNA-based therapeutics mediated by integrative lentiviral vectors and AAVs have become widespread in the market, RNA therapies are expected to be more versatile, and to cover a broad range of applications with minimal risks to treat or prevent a large variety of diseases. RNA-based therapeutics target applications in which a transient expression is safer to stimulate a cellular process, to modify a genetic sequence or to commit cells into a specific pathway.

Depending on the disease, delivered RNAs are designed to fit with the selected therapeutic strategy: gene-editing (CRISPR/Cas9 technologies, including Base editing), regenerative medicine, or immuno-oncology. RNAs optimisations depend on target cells, the need for an *ex vivo* or *in vivo* approach, gene(s) of interest, expression level, and duration requirement.

A game-changing RNA carrier called FlashRNA®(formerly LentiFlash) has been developed to overcome the current limitations of DNA delivery (AAVs and lentivectors) such as long-term DNA presence or genomic integration, and mandatory repeated administrations for multiple genes delivery which bring safety issues. FlashRNA® technology masters electroporation and LNPs by its own characteristics. It can deliver very specifically multiple RNAs species thanks to an engineered and patented new packaging method. Hence, due to a highly robust structure, RNAs are protected from degradation, they are delivered very efficiently into the cell cytoplasm, and they are directly available to be translated into proteins without any risk of cell damage or phenotype impairments.

In an innovative regenerative therapy approach, FlashRNA® is validated as the gene delivery system of choice to restore the lymphatic function of patients with secondary lymphedema (LD). LD is a multifactorial pathology with currently no treatment, appearing after breast cancer surgery. Preclinical results show that co-delivery of VEGFC and Apelin mRNAs by FlashRNA® abolished lymphedema and restored the lymphatic flow in the limb of a mouse model (Creff *et al.* EMBO Mol Med, 2024). This new treatment will be administrated to patients for a Phase I/II clinical trial called Theralymph that will be started beginning of 2025. FlashRNA® proves to be a game-changing gene therapy approach for the treatment of multigenic diseases.

These properties, and the ability to produce FlashRNA® using an existing cGMPs-compliant production platform, provide a very promising method for safe and efficient therapy in Human. It offers unprecedented safety considerations in combination with high efficiency compared to other therapeutic approaches.

P0954

Understanding and Improving TFAMoplex-mediated transfection with Interaction Proteomics

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Gene therapy is now offering new treatments for several diseases. However, delivering DNA into target cells still poses challenges due to its large size and negative charge. While viruses are efficient gene carriers, nonviral vectors are generally preferred for their safety and ease of production. They are, however, less effective. Our group recently reported a DNA transfection system relying on the human mitochondrial transcription factor A (TFAM) that is referred to as TFAMoplex. TFAM binds and condenses DNA into transfecting nanoparticles, offering a promising approach to address the low efficiency issue. The original TFAMoplex incorporated a bacterial phospholipase for endosomal escape and vaccinia-related kinase 1 (VRK1), which notably promoted transfection efficiency *via* an unidentified mechanism. This investigation aims at replacing VRK1 with dynein light chain proteins, specifically RP3, to directly link the complexes to the dynein motor complex, facilitating cytosolic transport. We verified the interaction between the resulting fusion protein TFAM-RP3 and dynein intermediate chains 1 and 2. Additionally, we implemented for the first time a proteomic-based assay to compare cytosolic protein interactions of various TFAMoplex variants, including the RP3-modified version and the original VRK1-containing system. Significant shifts in protein interactors were observed in the VRK1-containing TFAMoplex group, particularly involving nucleolar proteins. Utilizing this insight, we integrated one of these nuclear proteins, leucine-rich repeat-containing protein 59 (LRRC59), into the TFAMoplex, resulting in a notable enhancement of transfection efficacy compared to the RP3-modified system and similar levels to the original VRK1-containing version. This study not only enhances our understanding of the TFAMoplex system but also sheds light on the tremendous potential of protein engineering in designing effective gene delivery systems.

P0955

Novel Non-viral Biocompatible Nano Hydrogel for Gene delivery

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Here in, we have designed, synthesized, and characterized novel cross-linked monodispersed nanohydrogels (NHGs) with well-defined sizes ranging between 50–400 nm for nucleic acid packaging and delivery towards gene expression and regulation. The NHG's are obtained from mixtures of N-isopropylacrylamide, acrylonitrile, di-block, and tri-block jeffamine macro-monomers in the presence of a cross-linker and radical initiator. The mechanism of NHG formation includes the formation of a self-assembly obtained by heating the thermo- responsive monomer's mixture. The final size of the final NHG is dictated by the size of the intermediary self-assembly. Sizes are tuned by combining different ratios of the starting monomeric mixtures which upon heating from self-assemblies of varied sizes. Initiator is then added at high temperature and the polymerization gives place to the formation of NHGs. The obtained NHGs were chemically reduced to lead particles with highly positive zeta potential and low cell toxicity. The obtained reduced NHG's were extensively characterized including DLS, zeta potential, FTIR and AFM. The NHGs are highly biocompatible and can complex pDNA to form polyplexes devoid of cell toxicity as assessed by XTT assays. The in-vitro result reveals that the polyplex complexes have the ability of long-term gene expression. The in-vivo intramuscular and subcutaneous administration shows a delayed but prolonged expression of the marker gene. The new platform might be applied to vaccination, since it will certainly better protect nucleic acids and allow longer expression of the antigenic protein for more efficient vaccination.

P0956

Rationally designed guanidyl-rich poly(beta amino ester)s as non-viral vectors for protein therapeutics and CRISPR/Cas9 RNP-based gene editing therapy

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Poly(beta amino ester)s (PAEs) have been extensively utilized as non-viral gene delivery vectors in therapeutic applications, from cancer gene therapy to tissue engineering areas. While PAEs exhibit exceptional DNA delivery capabilities, their use in protein delivery remains limited. Similar to other cationic polymers, PAEs form nanoparticles with nucleic acids via electrostatic interactions, which are often insufficient for effective protein complexation due to the diverse surface charges of proteins.

In this study, we designed and modified PAEs by incorporating phenyl guanidine (PG) to enhance interactions between PAEs and proteins, facilitating intracellular delivery of various proteins *in vitro*. These proteins include saporin, bovine serum albumin (BSA), IgG, CRISPR/Cas9 nuclease and ribonucleoprotein (RNP) complexes, and beta-galactosidase, with molecular weights ranging from 30 kDa to 464 kDa and isoelectric points between 4.3 and 9. We first modified a linear PAE (LPAE) with the PG group to investigate its potential in functional cytosolic protein delivery. Using BSA as a model protein, we systematically compared the protein binding capacity, intracellular transport ability, endocytosis pathways, and endosomal escape efficiency of guanidyl-rich PAE (LPAE-PG) with the corresponding traditional LPAE. The PG modification facilitated multiple interactions, including electrostatic forces, salt bridges, and hydrogen bonds, resulting in superior protein binding and more efficient cell uptake. Notably, LPAE-PG demonstrated enhanced delivery of functional proteins such as saporin, beta-galactosidase, and CRISPR/Cas9 RNP, outperforming commercial protein transfection reagents like PULsin and Pierce Protein Transfection Reagent.

In GFP-positive HeLa cells, LPAE-PG delivered CRISPR/Cas9 RNP, achieving 19% targeted gene editing in polyclonal populations, comparable to the commercial CRISPR/Cas9 RNP delivery reagent, Lipofectamine CRISPRMAX, and three times more targeted indels than unmodified LPAE. To further improve CRISPR/Cas9 RNP delivery, we developed highly branched guanidyl-rich PAEs (HPAE-PG). These three-dimensional HPAE-PG, characterized by intricate structures and multiple terminal groups, incorporated more PG groups, thereby further enhancing their cytosolic protein delivery capacity. We evaluated the impact of branched monomer structure and branched ratios of HPAE-PG on buffering capacity, protein binding, intracellular delivery efficiency, cytotoxicity, and protein biofunction protection. Furthermore, HPAE-PG were tested for CRISPR/Cas9 RNP delivery, targeting *COL7A1* exon 80 deletion as a gene editing therapy for recessive dystrophic epidermolysis bullosa (RDEB). The HPAE-PG nanoparticles complexed with CRISPR/Cas9 RNP achieved 69% indels and 32% targeted *COL7A1* exon 80 deletion in HEK polyclonal cells.
The rationally designed guanidyl-rich HPAEs demonstrated robust capabilities for functional cytosolic protein and CRISPR/Cas9 RNP delivery. The successful targeted exon deletion observed in HEK and RDEB patient-derived skin cells underscores the potential of guanidyl-rich HPAEs as non-viral vectors in gene editing therapies for genetic diseases such as RDEB.

P0957

Lipid-based nanoparticles as game-changers in mRNA-mediated CRISPR-Cas engineering of B cells

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Delivery of mRNA to immune cells has emerged as a promising strategy for engineering and harnessing the therapeutical power of immune cells, especially T, natural killer and B cells. However, current methods, such as viral vectors or electroporation, have limitations in terms of safety, efficiency and scalability. Lipid-based nanoparticles evolved as an appealing alternative, offering a versatile platform for efficient and biocompatible mRNA delivery to these cells. Nevertheless, there is still a necessity for improving the performance and comprehending the underlying mechanisms of efficacy. Currently, there is a growing industrial and clinical interest in B cell engineering and subsequent adoptive B cell transfer, which already displayed good preclinical data. Efficacious, simple & scalable solutions to engineer B cells, particularly by utilization of CRISPR-Cas, remain to be uncovered and are a significant research priority. Thus, we aim to leverage the lipid nanoparticles (LNPs) for improved mRNA delivery to B lymphocytes and achieve therapeutic potency through CRISPR-Cas engineering. To achieve this, we assessed and further optimized our LNPs that we previously developed and described to have superior efficiency in transfecting NK cells. After validation that those LNPs achieved 100% GFP transfection efficiency without inducing toxicity in B cell lines, we exploited the Design Of Experiment approach by finely tuning production parameters to further improve the overall LNP potency. As a result, we obtained high and stable expression of GFP in the B cell lines. Then, we focused on the CRISPR-Cas system for B cell engineering with our model featuring GFP-expressing BL41 lymphoma cells. Cas9 mRNA and sgRNA targeted against the GFP gene were loaded into LNPs, resulting in more than 80% of GFP knock-out. Building on the promising results with cell lines, we next evaluated efficiency in primary B (pB) cells. Pre-activated cells treated with LNPs achieved over 90% transfection of pB cells that were positive for activation markers without notable mortality. This donor-independent efficiency was contrasted with the transfection failure of benchmark formulation, LNP SM102. Finally, as a proof-of-concept, we successfully delivered Cas9 mRNA and two sgRNAs encapsulated in LNPs to induce class switching recombination (CSR) in the antibody heavy chain locus of pB cells, showcasing the high potency of our approach.

Overall, our LNPs provide a scalable platform for highly efficient transfection of B cells, enabling a versatile utilization of the CRISPR-Cas system - a key approach to fully harness the therapeutical potential of B cells.

P0958

Stable gold nanoparticle-conjugated polyplexes for CRISPR-Cas9 delivery in CD34+ Cells

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Therapeutic potential of CRISPR-Cas9 could be greatly expanded by the development of simple, effective and safe delivery systems for Cas9 ribonucleoprotein (RNP) complexes, especially for hard-to-transfect cells like hematopoietic stem and progenitor cells (CD34+). We previously described a gold nanoparticle (AuNP) based CRISPR RNP delivery system (Shahbazi, R., Nature Materials, 2019) for non-electroporation mediated delivery to CD34+ cells. However, this system failed to deliver Cas9, the most commonly used CRISPR system. We have since established the physiochemical properties of Cas9 which compromise CRISPR-AuNP assembly and report a modified CRISPR-AuNP structure which can successfully load Cas9, but at the expense of polymer needed to successfully deliver CRISPR-AuNP cargo into CD34+ cells (Lane, D. bioRxiv, 2024).

This study evaluates the use of thiolated poly(ethyleneimine) poly(ethylene glycol) copolymers (PEI-PEG-SH) and gold nanoparticles (AuNP) for delivering Cas9 RNP complexes targeting the β-2-microglobulin (B2M) gene in primary human CD34+ cells. PEI-PEG-SH copolymers were synthesized to form polyplexes with Cas9 RNP complexes, leveraging the cationic nature of co-polymer to form polyplexes with negatively charged Cas9 RNP complexes at various amine to phosphate (N/P) ratios. While optimal gene editing efficiency of 10% indel formation at the B2M locus at an N/P ratio of 8 was confirmed with flow cytometry and MiSeq DNA sequencing, dynamic light scattering (DLS) revealed polyplex instability at all N/P ratios. Cell viability assays demonstrated minimal toxicity at the N/P ratio of 8, though higher N/P ratios resulted in increased cytotoxicity.

We hypothesized that stability of PEI-PEG-SH polyplexes could be restored by electrostatic binding with negatively charged AuNPs using copolymer thiol-gold chemistry. A 20 nm gold core solution was used to conjugate the polyplexes resulting in stable CRISPR-AuNPs with hydrodynamic diameters of 86.31 ± 1.90 nm, zeta potential of +1 mV and a polydispersity index of 0.258 ± 0.01 as confirmed by DLS. Cargo release followed by SDS-PAGE confirmed successful conjugation of nuclease onto AuNP. B2M CRISPR-AuNP conjugates were then tested in CD34+ cells. Flow cytometry and MiSeq DNA sequencing confirmed significantly increased levels of indel formation over mock treated sample, with up to 4.87 ± 0.98% of sequence reads displaying indels, with no adverse effect on cell viability. In vivo testing of this nanoparticle in humanized NBSGW mice is in progress. This study contributes to the ongoing development of biocompatible and efficient, non-electroporation-mediated delivery methods for gene therapy research and clinical applications, particularly for difficult-to-transfect primary cells like CD34+ cells.

P0959

microRNA-758 inhibits migration of metastatic breast cancer cells

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Metastatic breast cancer is the most advanced form of breast cancer. Although advances in medicine have improved outcomes and provided more treatment options, patients with advanced stages remain incurable and have poor survival rates. MicroRNAs (miRs) are post-transcriptional regulators of gene expression and previous work by our research group identified miR-379 as a potent tumour suppressor in breast cancer. miR-379 is part of a large miR-379/656 cluster and this study aimed to investigate the role of another microRNA within this cluster, miR-758, in the breast cancer setting. MDA-MB-231 breast cancer cells were stably transduced with a lentiviral vector carrying non-targeting control (NTC) sequence or mature miR-758 along with green fluorescent protein (GFP) to generate MDA-NTC and MDA-758 cells. Transduction success was confirmed by fluorescent imaging and analysis of miR-758 expression by quantitative real-time PCR (qPCR). Transwell membranes with a pore size of 8 µm were employed to assess the migration capacity of MDA-NTC, MDA-758 and wild type cells in response to chemoattractants. Migrated cells were fixed, stained with crystal violet and counted using Image J. MTS assay was performed on MDA-NTC and MDA-758 cells to investigate the impact of miR-758 on cell proliferation. In addition, an initial tubule formation assay was employed to investigate the impact of miR-758 overexpression on the ability of MDA-MB-231 cells to stimulate angiogenesis. MDA-MB-231 transduced with NTC or miR-758 vectors successfully expressed GFP fluorescent signal, and qPCR analysis revealed a 180-fold increase in miR-758 expression in MDA-758 compared to MDA-NTC cells. There was no significant change in proliferation between the miR-758 enriched cells compared to control populations. Transwell results showed that the number of migrated cells in the MDA-NTC group (294 cells ± 10 cells) was significantly higher than that in the MDA-758 group (193 cells ± 26 cells). MDA-758 cells demonstrated a 34% reduction in migration (P=0.04) towards chemoattractants across transwell inserts in comparison to MDA-NTC cells. The initial results of the tubule formation assay suggest conditioned media of MDA-758 cells had a reduced ability to stimulate tubule formation compared to the conditioned media of control cells, with analysis of secreted factors ongoing. MDA-MB-231 cells were successfully engineered to stably express miR-758. Although no impact on cell proliferation was observed, enrichment with miR-758 resulted in a significant decrease in migratory capacity of this highly invasive cell population. Combined with results suggesting decreased capacity to stimulate angiogenesis, this exciting data supports a potential tumour suppressor role for miR-758. Further studies will be performed to determine the specific mechanism of action of this microRNA.

P0960

Investigation of endothelin-1 regulation in breast cancer

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Endothelin-1 (EDN1) is primarily secreted by endothelial cells in blood vessels where it has a potent vasoconstricting or vasodilating effect depending on specific receptor activation. Elevated circulating EDN1 in breast cancer patients has been associated with lymph node and distant metastasis. EDN1 has also been implicated in bone metastasis, where tumour-derived EDN1 was shown to promote osteoblastic proliferation through binding with Endothelin Receptor Type A

(EDNRA). microRNA-379 (miR-379) is believed to be a potent tumour suppressor, and has a predicted binding site in the 3' UTR of EDN1. The aim of this study was to determine whether miR-379 plays a role in regulation of EDN1 in breast cancer. Breast cancer cell lines [Human: MDA-MB-231, HCC1954; Murine: 4T1] were transduced with lentivirus to express miR-379 or a non-targeting control (NTC) sequence. RNA was extracted from cells and tissue xenografts, and RQ-PCR performed targeting EDN-axis members: EDN1, endothelin converting enzyme 1 (ECE1), endothelin receptor type A and B (EDNRA, EDNRB). ELISA was used to detect EDN1 secreted in conditioned media from the transduced cells at 24, 48, and 72 hour time points. Following establishment of HCC1954-379 or -NTC, and 4T1-379 or -NTC tumours *In Vivo*, tissues were harvested and immunohistochemistry (IHC) targeting Prepro-EDN1 was performed to detect changes in protein expression in the presence of miR-379. EDN1 was confirmed to be present in all cell lines by RQ-PCR, with no significant change at a mRNA level in the presence of elevated miR-379. Expression of EDN-axis genes by PCR varied between cell lines. EDN1 and ECE1 were robustly expressed in all cell lines, with highest EDN1 in HER2 amplified HCC1954 cells, and highest ECE1 detected in triple negative MDA-MB-231 cells. MDA-MB-231 had robust expression of both receptors, while HCC1954 cells had negligible receptor expression. Both receptors were undetectable in murine 4T1 cells, but were strongly expressed in 4T1 tumours likely due to receptor presence in infiltrating vasculature. miR-379 enrichment had no impact on EDN1 secretion *in vitro* at 24, 48, and 72 hour time points. The amount of EDN1 secreted after 72 hours varied significantly between cell lines: MDA-MB-231 secreted the lowest level at 4.5 +/- 0.4 pg/ml (Mean +/- SEM) despite having the most robust EDN1 mRNA expression, followed by 4T1 at 53.0 +/- 8.7 pg/ml, and HCC1954 with the highest EDN1 secretion at 319.1 +/- 47.9 pg/ml. Both 4T1 and HCC1954 *ex-vivo* tumours stained for EDN-1 by IHC showed a significant reduction of Prepro-EDN1 in tumours with enriched miR-379 compared to respective control tissues. The initial data presented from *in vitro* and *in vivo* experiments suggests that miR-379 may play a role in regulation of EDN1 in breast cancer. Understating the interplay between genes involved in EDN1 processing and uptake will further elucidate the functional relevance in disease progression.

P0961

Comprehensive evaluation of T7 polymerase for enhanced yield and quality in mRNA production

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messenger RNA (mRNA) drug modalities are a revolutionary breakthrough in medicine, offering the potential to treat a wide variety of rare and infectious diseases. ReciBioPharm, contract with MIT, follows the largest award from FDA's Center for Biologics Evaluation and Research office (CBER) to MIT, enabling next generation mRNA manufacturing capacity and providing faster readiness and reaction to new pandemic threats. One major effort of the ground-breaking initiatives is to systematically identify critical raw materials during mRNA production and scientifically understand their impact on the final quality of these drug products. In vitro transcription (IVT) is an enzymatic process used in the synthesis of mRNA products. In a typical IVT process, a RNA polymerase is responsible for the transcription of RNA from a DNA template. While T7 polymerase is the most common used in both research and commercial purposes and there are multiple offerings of both wild type and engineered T7 polymerases in the market, so far there is no systematic studies to understand the performance of these commercially available T7 polymerases. Herein, we conducted a comprehensive study to characterize total 19 of T7 polymerases including 4 wild types and 15 modified ones. Briefly, a high throughput screening

(HTS) workflow was established to enable >1500 IVT runs with subsequent critical analytics including yield, purity, dsRNA and potency; these DOE based optimizations were followed by subsequently lot-to-lot variations, and scalability studies; the desired process range for each T7 was defined and some high potential process parameters was proposed to offer >15g/L mRNA yield with >95% purity and minimum dsRNA (below dsRNA ELISA LOD). The outcomes as a T7 knowledge library with deep profiling their performance & cost analysis was well established, which will accelerate the deployment not only of mRNA vaccines for rapid response against future pandemics but also of novel mRNA therapeutics.

P0962

Thermostable Hermes™ lipopolyplex nanoparticles for the delivery of mRNA produced from synthetic, enzymatically produced DNA

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Manufacture of GMP grade DNA is a major bottleneck in mRNA production with a worldwide lack or capacity and long lead times of plasmid DNA. Additionally, the thermostability of mRNA lipid nanoparticle (LNP) vaccines is problematic, with reported shelf-lives of just 1 month at 2-8°C, or hours at room temperature. Combining our synthetic DNA and Hermes™ platforms, 4Basebio aims to address these issues. Synthesis of opDNA™ is fully enzymatic facilitating large-scale production of linear DNA devoid of bacterial backbone with improved yields over traditional plasmid fermentation processes. Enzymatic linearisation of opDNA™ is not required prior to vitro transcription (IVT) and, continuous polyA tails >120 bp, can be coded into the template. Meanwhile, Hermes™ nanoparticles exploit a cationic ligand, combined with traditional lipids, to drive payload condensation and extend shelf life at fridge temperature.

IVT synthesis of mRNA using an opDNA™ template achieves significantly higher yields than linearised plasmid, with equivalent capping efficiency and dsRNA impurities, for constructs ranging from 1.5-12kb. Likewise, proinflammatory cytokine/chemokine levels in isolated primary human PBMCs are comparable to mRNA produced from linearised plasmid. Further, when encapsulated in Hermes™ nanoparticles, mRNA encoding firefly luciferase from an opDNA™ template achieves comparable bioluminescence to linearised plasmid in HEK293T cells and in mice following intramuscular delivery.

To drive long term storage stability, the relationship between typical lipids used in LNPs and a 5th cationic ligand has been extensively investigated in Hermes™ formulations. Using a DOE approach, lipid and ligand molar ratios were optimised to favour thermostability. Interestingly, after 12 weeks at 4°C, linear regression analysis found that encapsulation efficiency (EE) positively correlated with ligand ratio, independent of the ionizable lipid type or the molar ratios of the four lipid components. In total, 5 Hermes™ formulations maintained a stable size, PDI and EE (change of <15% between 0-12 weeks) and all formulations at the highest ligand ratio maintained an EE >95%. Conversely, literature values widely report a drop in EE after 4 weeks for conventional LNPs.

In the therapeutic setting, Hermes™ formulations encapsulating 4Basebio mRNA encoding flu antigen demonstrated comparable antibody titres to a commercial LNP control following a single prime dose delivered intramuscularly to mice. Similarly, when encapsulating a neoantigen mRNA

payload, Hermes™ formulations prevented the development of tumours in 6/7 mice following 3 doses, 28 days apart, and a tumour challenge on day 52.

Therefore, IVT synthesis of mRNA from an opDNA™ template is both efficient and efficacious, lending itself to large-scale gene therapy or vaccine applications. Further, the inclusion of a cationic ligand is shown to enhance stability at 4°C. Combined with the demonstrated therapeutic efficacy, this thermostability may preclude the cold chain storage requirements of conventional LNPs, the cost of which can be prohibitively expensive, particularly in developing nations.

P0963

Mitochondria as versatile vehicles for therapeutic gene delivery

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Ischemia reperfusion injury can be reduced by transplantation of the affected tissue with purified mitochondria – known as Therapeutic Mitochondria Transplantation (TMT). The observed local distribution pattern of mitochondria after intra-arterial injection along with their efficient uptake into cells in the target organ motivated us to investigate whether these organelles could be used as a delivery vehicle for gene therapy applications.

A key observation has been that limits to payload size and number described for most viral vectors do not apply to mitochondria. We demonstrated that mitochondria can carry more than 50 DNA molecules per particle, and that plasmids of 7kb can be delivered into cells efficiently. Investigations to determine the upper limit of payload capacity are ongoing.

Mitochondria complexed with various oligonucleotides such as mRNA, plasmid, or siRNA showed efficient uptake by cells *in vitro* and *in vivo* and generate their cognate proteins or reduce expression of the target proteins. For applications that require expression of DNA and RNA simultaneously, we demonstrated that when mRNA and plasmid DNA are complexed with mitochondria, co-expression of both mRNA transgene and plasmid expression can be achieved efficiently.

Expression of several different reporter proteins was detected up to 3 days for mRNA payload and up to 3 weeks in rapidly dividing cells for plasmids, providing a window of transient delivery and duration of expression of nucleotide-based therapeutics based on the type of nucleotides used. Additional *in vitro* studies indicated that the mitochondria used for nucleotide delivery are detectable only up to day 6 while reporter protein expression persisted up to 21 days.

Payload uptake could further be improved by addition of targeting moieties. A cell line expressing the relevant antigen exhibited a circa 7-fold increased uptake and 3-fold increase in protein expression, after treatment with mitochondria:mRNA complex coupled to an antibody, compared to complexes lacking the additional antibody.

Initial *in vivo* experiments demonstrated uptake of mitochondria and expression of their mRNA cargo by porcine kidney cells following renal artery injection, delineated by presence of human mitochondrial DNA as well as clear mCherry expression. In mice, tail vein and direct injection of complexes encoding luciferase showed luciferase activity in multiple organs, including the kidney. This is of significant interest given the scarcity of approaches that allow safe delivery of genetic material to major internal organs other than the liver.

We demonstrated that purified mitochondria can be complexed with nucleotides encoding multiple reporter proteins, and that these complexes can drive the expression of reporters both *in vitro* and *in vivo*. Further modifications of the complex with targeting moieties increased transgene expression. These initial observations show that mitochondria can be used as an efficient and versatile vehicle for gene delivery with potential to bring gene therapy to organs traditionally refractive to gene therapies such as the kidney.

P0964

Enhancing Natural killer cells proliferation and cytotoxicity using Imidazole-based lipid nanoparticles encapsulating interleukin-2 mRNA

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Messenger RNA applications have undergone unprecedented applications - from vaccination to cell therapy. Natural Killer cells are recognized to have a significant potential in immunotherapy. NK -based cell therapy has drawn attention as allogenic graft with a minimal graft-versus-host risk leading to easier “off-the-shelf” production. NK cells can be engineered with either viral vectors or electroporation, involving high costs, risks, and toxicity, emphasizing the need for alternative way as mRNA technology. We successfully developed, screened, and optimized novel lipid-based platforms based on imidazole lipids. Formulations are produced by microfluidic mixing and exhibit a size around 100 nm with a polydispersity index less than 0.2. They are able to transfect NK-92 cells, KHYG-1 cells and primary NK cells with high efficiency without cytotoxicity while Lipofectamine Messenger Max and D-Lin-MC3 LNP based formulations do not. Moreover, the translation of non-modified mRNA was higher and more stable in time compared to modified one. Remarkably, the delivery of therapeutically relevant IL2-mRNA resulted in extended viability together with preserved activation markers and cytotoxic ability of both NK cell lines and primary NK cells. Altogether, our platforms feature all prerequisites needed for the successful deployment of an NK-based therapeutic strategies.

P0965

Development of novel luminescent assays for sensitive and specific quantitation of double-stranded RNA in mRNA therapeutics

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Double-stranded RNA (dsRNA) is a byproduct and contaminant of in vitro transcription (IVT) products. dsRNA is highly immunogenic and can be detected by several intracellular or endosomal sensors, leading to inflammation, translation inhibition, and cell death. Existing methods to detect dsRNA in mixed solutions lack quantitation, specificity, and sensitivity.

We have developed two novel assay systems for dsRNA detection and quantitation using bioluminescence**.** dsRNA Detection Assay using NanoBiT® technology (Lumit® dsRNA Detection Assay) detects dsRNA using dsRNA binding domains (DRBDs) genetically fused to SmBiT and LgBiT. Dimerization of the BiT-DRBDs on dsRNA induces complementation of NanoBiT® luciferase and generation of light. This assay provides sensitive quantitation of dsRNA in mixed solutions and overcomes the inadequacies of existing ELISA and blotting technology. It is easy to implement and features a simple add-mix-read protocol with no immobilization or wash steps. The second assay for specific detection of dsRNA is a cell-based TLR3-activation reporter bioassay. Upon binding of dsRNA to TLR3 in the endosomes, the activation of TLR3-responsive promoter drives the reporter luciferase expression and light output. This assay enables MoA-relevant measure of cellular response to dsRNA in encapsulated mRNA preparations. It's also easy to implement and can be used in "Thaw-and-Use" format, no cell culture required.

P0966

Lipid nanoparticle delivery of multiplexed siRNAs for a functional HIV cure

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During primary infection, the genome of Human Immunodeficiency Virus (HIV) is permanently integrated into the host cell chromosomal DNA, and a latent viral reservoir is established predominantly in $CD4^+$ T cells. While antiretroviral therapy has transformed HIV into a chronic manageable illness, the viral reservoir rebounds if treatment is ever stopped, and thus a person's control of HIV is dependent on life-long treatment. This reservoir continues to be one of the major barriers to achieving a HIV cure ~40 years since the virus emerged.

The block and lock cure strategy aims to permanently silence HIV, by blocking virus transcription and locking the reservoir in a state of deep latency. The use of short-interfering RNA (siRNA) to induce epigenetic silencing, which target conserved regions of HIV genome, is an attractive approach to achieving a broad-spectrum cure. In addition, the increasing success and optimisation of *in vivo* RNA delivery platforms, such as lipid nanoparticles, will facilitate the clinical translation of siRNA for novel applications.

To address this ambition, a panel of siRNAs were designed to highly conserved regions of the HIV promoter and the antiviral efficacy determined *in vitro* by measuring infection via flow cytometry and viral mRNA by RT-qPCR. Antiviral siRNAs were encapsulated in a lipid nanoparticle using a NanoAssemblr® Ignite and characterised for size and encapsulation efficiency. HeLa T4+ cells were transfected with siRNA-LNPs and the antiviral effectiveness was determined by measuring viral mRNA by RT-qPCR and protein by the Reverse transcriptase assay. Functionalisation of lipid nanoparticles by bispecific antibodies was employed for targeted delivery to Jurkat E6 (T cell line), U937 (monocytic cell line) and human primary $CD4^+$ T cells.

Experimental validation confirmed that several siRNA were able to provide significant antiviral protection against seven HIV Subtypes *in vitro* demonstrating pseudovirus mOrange reductions of up to 65% ($P<0.05$), and that multiplexing 3 siRNA together maintains antiviral efficacy ($P<0.05$) and creates a broad-spectrum antiviral with >99% sequence coverage of all circulating HIV-1 Group M subtypes globally. siRNA delivery via LNP (~90 nm in size, ~90% encapsulation efficiency) resulted in a significant reduction of viral gag mRNA levels of up to 85% ($P<0.05$) and reverse transcriptase protein up to 50% ($P<0.05$) compared to controls *in vitro* in HeLa T4+ cells. In addition, pseudovirus GFP reductions of up to 55% in Jurkat E6 and U937 cells were observed ($P<0.05$) and bispecific antibodies achieved targeting of LNPs *in vitro*, as measured by flow cytometry, demonstrating increased cell association in a receptor specific manner.

We show that LNPs, which are already clinically approved for the delivery of RNA-based therapies, possess enormous potential as a delivery platform for delivering a functional HIV cure. Additionally, the potential to deliver multiplexed siRNAs via LNPs could further develop an antiviral that is effective independent of HIV subtype.

P0967

Hermes™ lipopolyplex nanoparticles for synthetic, enzymatically produced linear hpDNA™ delivery

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Non-viral mRNA vaccines have been hugely successful, but issues remain surrounding mRNA instability and cold chain storage requirements, as well as manufacture of GMP grade DNA templates for mRNA production. Theoretically, DNA payloads have the advantages over mRNA of increased transgene persistence and superior stability. However, due to the barriers of nuclear uptake and immunogenicity, delivery of DNA payloads is underexplored. 4Basebio has a fully enzymatic DNA manufacturing process that is size and sequence independent, enabling gram scale, cell-free, GMP manufacture of DNA devoid of any bacterial sequence with expedited turnaround times. Here, Hermes™ nanoparticles comprising a cationic ligand, as well as traditional lipids, to drive payload encapsulation, are optimised for delivery of 4basebio's synthetic DNA constructs.

First, a one-step microfluidics method for combining lipids, ligand & payload was developed. The Hermes formulations had a comparable size, PDI and encapsulation efficiency (EE) as compared to a conventional LNP and, the incorporation of a fluorescently labelled ligand was confirmed at a density of 0.734 ligands/100 nm^2 (106 per nanoparticle) using nano flow cytometry (NanoFCM). Next, lipid & ligand molar ratios were optimized for hpDNA™ encapsulation. Interestingly, lipid compositions optimal for mRNA delivery were found insufficient for DNA payloads. Whilst lipids used in conventional LNPs produced small, uniform, stable nanoparticles when combined with

ligand and mRNA, the same formulations for hpDNA™ were unstable, precipitating following dialysis in PBS. Using an iterative DOE approach, stable hpDNA™ formulations with favourable biophysical characteristics, EE and *in vitro* transfection were identified.

To test the *in vivo* efficacy of the optimised formulations, luciferase reporter hpDNA™ was encapsulated and delivered intramuscularly to mice at a dose of 0.4mg/kg. An improvement in bioluminescence of >1-log over a conventional LNP formulation was observed. Moreover, the optimized Hermes™ formulation encapsulating hpDNA™ demonstrated higher luciferase expression than the same formulation encapsulating plasmid DNA at both equimass and equimolar dosing, demonstrating the merit of combining both 4basebio's DNA and Hermes™ platforms.

Finally, to evaluate whether this increased expression was sufficient to drive therapeutic efficacy, hpDNA™ encoding neoantigens was delivered intramuscularly with the optimised Hermes™ formulation, as compared to an electroporation control. Three doses of Hermes™ 28-days apart completely prevented the development of tumours in 2/7 mice following a tumour challenge at day 52, which was comparable to the electroporated hpDNA™. Further, survival rate was significantly greater in Hermes™ treated mice compared to untreated mice; 83.3% of Hermes™ hpDNA™ treated mice survived compared to 0% in the untreated group and 50% in the electroporated hpDNA™ group.

Therefore, we show that by combining next generation DNA and lipopolyplex platforms, DNA delivered with non-viral vectors can be efficacious *in vivo*. With the superior stability of DNA payloads and the fast, scalable manufacture of synthetic DNA, 4Basebio's platforms are ideally suited to vaccine and personalised medicine applications.

P0968

Correction of argininosuccinate lyase deficiency by precision CRISPR base editing

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Argininosuccinate lyase deficiency (ASLD [MIM: 207900]) is a recessive metabolic disorder caused by variants in *ASL*. In a critical step in the urea cycle, ASL breaks down argininosuccinate (ASA) into arginine and fumarate. The Finnish founder variant (RefSeq# NM_000048.4 (*ASL*): c.1153C>T, p.Arg385Cys) is present in homozygosity or compound heterozygosity in 70% of the ASLD individuals in Finland and it causes a severe form of the disease, leading to hyperammonemia, neurological injury, and even early death. The current treatments involve a strict low-protein diet, arginine supplementation, nitrogen scavenging, and in some cases, liver transplantation. However, the current palliative care options are unsatisfactory, leaving an unmet need for improved, efficient therapies. Here, we show the potential of a lipid nanoparticle-mediated CRISPR approach using adenine base editors (ABEs) for ASLD treatment.

To model ASLD, we first generated ABE-corrected hiPSC lines from biopsies of two individuals homozygous for the Finnish founder variant. We then differentiated the hiPSCs into hepatocyte-like cells, which showed a more than 1,000-fold decrease in ASA levels compared to isogenic non-edited cells. To assess delivery methods compatible with clinical applications, we tested three

different FDA-approved lipid nanoparticle (LNP) formulations to deliver the ABE-encoding RNA and the sgRNA targeting the *ASL* variant. This approach efficiently edited the ASL variant in fibroblasts with no apparent cell toxicity and minimal off-target effects. Moreover, the LNP treatment resulted in the restoration of ASL enzyme activity and a significant decrease in ASA, reaching the levels of healthy donor fibroblasts. Our work describes a highly efficient approach to correct the disease-causing *ASL* variant, restoring the function of the urea cycle. This method relies on an RNA vector delivered by LNPs, which is compatible with clinical applications, improves its safety profile, and allows for scalable production.

P0969

A new linear plasmid process platform enabling small scale GMP production with significantly short lead time, high titer, superb polyA stability dedicating to nuclei acid production

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mRNA vaccines have been demonstrated as a powerful alternative to traditional conventional vaccines because of their high potency, safety and efficacy, capacity for rapid clinical development. The overall CMC production process of mRNA vaccines can be divided into three modules: pDNA production, mRNA production, and LNP encapsulation. While tremendous efforts have been dedicated into mRNA and subsequent LNP encapsulation process development, plasmid process has not been improved significantly and still heavily relied on the traditional approaches that was developed for viral vector applications. While it deliveries the DNA template for mRNA production, the tradition plasmid production has become the bottleneck for the mRNA vaccine applications due to its long lead time and high cost for both process development and manufacturing in its supercoil isoform and later linearization and difficulty in maintain the integrity of long coded polyA unit. At ReciBioPharm, we have developed a new plasmid platform dedicating to nucleic acid application. First, a systematic upstream optimization in cell bank, batch strategy, fed-batch strategy was executed to increase plasmid titer up to 1.5g/L fermentation batch size while maintain the desired full length polyA up to 120A. Second, linearization step was integrated as part of downstream process to reduce 30% unit operation and increase total recovery rate to 40% while reaching all required impurity specifications. This new platform has been well demonstrated with various plasmid sizes from 4000 to 9000bp and different polyA tail length from multiple DNA constructs. Such high titer can offer >500mg final linear plasmid from a 3L GMP batch size, which is enough to meet most of Phase I/II needs for mRNA vaccine clinical trials. Comparing to current 30L batch size based plasmid process in the market, it is no doubt that this new linear-dedicated plasmid process will significantly deployment not only of mRNA vaccines for rapid response against future pandemics but also of novel mRNA therapeutics to patients around the world.

P0970

Evaluation of the protein-based transfection agent TFAMoplex for *in vivo* DNA delivery

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Compared to viral vectors, non-viral gene delivery systems have gained attention in the past decades due to their low immunogenicity, independence of the size of transgenic DNA cargo, safety and flexibility in design. However, their limited stability in biologically relevant conditions and often non-specific interactions with target cells have led to a suboptimal therapeutic efficacy. In our previous research, the human mitochondrial transcription factor A (TFAM) was repurposed for plasmid DNA (pDNA) delivery. TFAM displays high DNA binding affinity independently of the sequence, which causes the DNA to be compacted into nanometer-sized complexes, called TFAMoplexes. To improve the transfection efficiency, TFAM was linked to additional proteins through genetic fusion. More specifically, TFAM was connected to phospholipase C (PLC) from *Listeria monocytogenes*, which hydrolyses phospholipids of the endosomal membrane at acidic pH, leading to an enhanced endosomal escape of the complex. Secondly, human vaccinia-related kinase 1 (VRK1) was fused to TFAM with the aim of inhibiting cytoplasmic DNA recruitment to the endoplasmic reticulum. Lastly, two cysteine point mutations in the TFAM homodimerization site stabilized the complexes, which allowed them to be formed in pure fetal bovine serum (FBS) and transfect HeLa cells using eGFP pDNA with an efficiency of approx. 30 % after 30 min incubation in FBS.

Next, in preparation of the *in vivo* evaluation of the TFAMoplexes in BALB/c mice, endotoxins were removed from PLC-TFAM and TFAM-VRK1 protein batches using a poly-L-lysine based endotoxin removal column. The combined endotoxin load post removal fell below the limit of 5.5 EU/mL as determined by the Limulus amoebocyte lysate (LAL) assay. The protein concentration and transfection efficiency before and after endotoxin removal were compared. A maximal decrease of 13 % in protein concentration was noted, while no significant differences could be detected in transfection efficiency. A viral contamination test of the TFAMoplexes by PCR analysis showed that this formulation did not contain any of the 13 tested murine viruses. Transfection experiments using TFAMoplexes combined with Firefly luciferase pDNA and bioluminescence imaging demonstrated that the complexes are able to transfect HeLa cells with different types of reporter genes. However, when using freshly isolated murine serum instead of FBS for TFAMoplex formation and transfection, an almost complete loss of transfection efficiency was seen, both in HeLa and C2C12 cells. After complex preparation in phosphate buffered saline (PBS) and transfection in FBS, the efficiency decreased to approx. 10 %. These data suggest that components from the FBS play an important role in TFAMoplex formation and potency. Together, these findings brought important insights for further optimization of the TFAMoplex formulation for upcoming *in vivo* gene delivery experiments.

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P0971

Intracellular trafficking of a protein-based transfection system

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Compared to viruses, non-viral gene delivery systems have a higher loading capacity and are considered safer. However, limited knowledge about the intracellular trafficking of non-viral transfection systems hampers their progress towards achieving higher efficiency. In our group, a protein-based transfection agent based on the DNA-binding protein Mitochondrial Transcription Factor A (TFAM) was recently introduced. TFAM forms nanoparticles of ~100 nm in diameter when complexed with DNA. It can be fused to the functional proteins phospholipase C (PLC) and vaccinia-related kinase (VRK1) to produce an efficient transfection agent. This protein-based transfection system named “TFAMoplex” was improved by fusing the DNA-binding domain of the transcription factor C-AMP response element binding protein (CREB) to TFAM-VRK1. CREB is a human protein that binds DNA inside the cytoplasm and is thought to enhance nuclear uptake. Hence, we hypothesized that the additional DNA-binding domain stabilizes the protein-DNA complex and increases transfection efficiency by promoting nuclear uptake. To understand the DNA delivery process at the cellular level, we compared the TFAMoplex, with and without CREB, to Lipofectamine. First, we studied the association of the transfection systems to HeLa cells by flow cytometry and confocal microscopy using Cy3-DNA. Second, we made use of a cell line, stably expressing EGFP-labeled barrier-to-autointegration factor (BAF) to study the fate of the DNA inside the cytoplasm. BAF is known to cluster transfected DNA immediately after cytosolic delivery. Third, mScarlet gene expression was measured one day after transfection via flow cytometry to quantify successfully transfected cells indicating effective nuclear delivery. The association of Cy3-DNA with the cells was highest for cells transfected with the TFAMoplex containing CREB. Cells associated with Lipofectamine showed more than 3x brighter mean fluorescence intensity than that of the TFAM-based transfection systems. To differentiate between intracellular and extracellular localization of the transfected DNA, Cy3-DNA that remained on the cellular membrane was counter-stained with an anti-Cy3 antibody and imaged on a confocal microscope. Colocalization analysis revealed that the largest fraction of DNA was localized on the cell surface for all transfection systems after 0.5 and 2 h. On the intracellular level, colocalization analysis of EGFP-BAF clusters with labeled DNA confirmed, that all BAF clusters contain the transfected DNA. Time-lapse imaging showed that cluster formation started approx. 1 h after transfection. Transfection under the challenging conditions (100% FBS, 0.5 h incubation, 100 ng DNA per $2x10^5$ cells) was highest for TFAMoplex bearing CREB and lowest for Lipofectamine. In summary, TFAMoplex with CREB has the highest transfection efficiency and strongest association to the cellular membrane. However, at this stage, it is not possible to correlate transfection efficiency with the intracellular trafficking of the tested non-viral gene delivery systems. This project has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation program (grant agreement No 884505).

P0972

Lipid Nanoparticles: from the laboratory to a pilot scale production

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Non-viral vectors (NVVs) have recently attracted renewed interest due to their success to tackle down infectious diseases (COVID). Their potential goes beyond infectious diseases, and promising results have been shown in gene and cell therapy. On top of that, NVVs are considered safer than their viral analogues and can potentially lower the production costs of the manufacturing of therapies.

Although there are different kinds of NVVs (liposomes, polyplexes with polymers or peptides, lipid nanoparticles), the most advanced ones generally rely on the stabilisation of the negative charges of the nucleic acids (mRNA, siRNA, plasmids…) with external positive charges by electrostatic interactions. In that sense, materials and small molecules bearing amino groups are easily protonated in a reversible manner at low pH and providing with positive charges to the systems.

Lipid nanoparticles (LNPs) have emerged as the principal frontrunner among the NVVs. They are based on a mixture of different modified lipids that encapsulate the therapeutic nucleic acids to increase their stability and target the desired tissues in vivo. The lipidic part is usually composed of i) an ionisable lipid (favours the endosomal escape within the cells), ii) cholesterol (controls their rigidity), iii) a helper lipid (favours stability and fusogenicity) and iv) a PEGylated lipid (enhances the colloidal stability and reduces the immune system recognition). Small modifications on the structure of the lipids can dramatically condition the in vivo behaviour of the LNPs. The efficacy of the LNPs is not only determined by the nature of their components, but also by the relative ratios among them (typically ionisable lipid: cholesterol:helper lipid:PEGylated lipid are 50:38.5:10:1.5, respectively). Therefore, a fine control over the nature of the lipids and their respective concentrations must be tuned to obtain successful LNPs.

A successful LNP formulation must be scalable and easily transferred to a manufacturing process. This is not always straightforward, since many LNPs formulations fail to reach the market due to the technical limitations observed when the process is scaled up. For example, the mixing step must ensure self-assembly in an efficient manner to obtain LNPs within the requirements and the purification by tangential flow filtration (TFF) is not always feasible for all LNP formulations.

Here, a fully scalable LNP-manufacturing process is shown based on different steps: i) preparation of solutions, ii) production of LNPs by flash nanoprecipitation, iii) purification and concentration by tangential flow filtration (TFF), iv) addition of cryoprotectant for final formulation, v) sterile filtration and vi) filling into vials. Based on the lab-scale protocol and the adaptations made for the scaled-up process, a quality control strategy (QC) was defined. It covers the raw materials acceptance, passing throw the different manufacturing steps (in-process controls), and the final product quality control. This GMP-like scale-up can be easily transferred to a CDMO.

P0973

Therapeutic Delivery of Mesenchymal stem cell-derived small extracellular vesicles for treating senescence-associated related diseases

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Small extracellular vesicles (sEV) derived from mesenchymal stem cells (MSC) have garnered significant interest for their potential in therapeutic applications, particularly for treating senescence-associated diseases. These vesicles are nano-sized particles that facilitate intercellular communication by transporting bioactive molecules, including proteins, lipids, and nucleic acids, such as microRNAs (miRNAs). miRNAs are small, non-coding RNA molecules that play critical roles ~~in~~ regulating gene expression and maintaining cellular homeostasis. In the context of senescence-associated diseases, characterized by the accumulation of senescent cells that contribute to aging and various chronic conditions like inflammation, MSC-derived sEV offer a promising avenue for therapy. They can deliver specific miRNAs to senescent cells, modulating their behavior and potentially reversing or mitigating the deleterious effects of cellular senescence. By targeting the molecular pathways involved in cell aging and inflammation, MSC-sEV carrying therapeutic miRNAs could improve tissue regeneration and overall function, highlighting their potential as a novel treatment strategy for age-related diseases. We generated functional EVs loaded with miR-4436b-5p and miR-30a-5p, which are involved in cell death and inflammation, respectively. Firstly, we isolated and characterized sEV from umbilical cord stromal-derived MSCs using Nanoparticle Tracking Analysis (NTA), Transmission Electron Microscopy (TEM), and immunoblotting. After that we loaded the miRNAs (miR-4436b-5p and miR-30a-5p) using sonication, electroporation, click chemistry, and ExoFect. The efficiency of miRNA loading was evaluated using qRT-PCR. Our results demonstrated the efficient miRNA loading was achieved using sonication, electroporation, and ExoFect, showing potential for modulating cellular behavior and mitigating the effects of cellular aging. In conclusion, our study demonstrates the efficacy of MSC-derived sEV as delivery vehicles for therapeutic miRNAs in treating senescence-associated diseases. These findings highlight the promise of MSC-derived sEV in regenerative medicine and age-related disease therapy.

P0974

A Versatile, Automated Assay for Same-Section Spatial Visualization of Point Mutation, Small RNA, mRNA, Protein and Protein-Protein Interactions in Cells and Human Colon Cancer Tissue Using Improved RNAscope Chemistry

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Non-clinical biodistribution studies are increasingly sought by regulatory agencies to understand the in vivo distribution, persistence and clearance of gene therapy products at the site of administration and in target and non-target tissues. We report a one-of-kind spatial multiomics assay that integrates the visualization of miRNAs/ASOs/siRNAs, exonic regions, point mutations, mRNAs, proteins, and protein-protein interactions in the same section using a tyramide signal amplification (TSA)-based protease-free chemistry. To generate proof of concept, we used a modified RNAscope chemistry for robust detection sensitivity of exon junctions and point mutations to integrate findings from multiple studies that detail the malignant cancer progression mechanisms in BRAF V600E mutant cell lines and colon cancer tissues:
1. Increased mitogen activated protein kinase (MAPK) signaling in the presence of BRAF V600E mutant
2. Increased MAPK signaling leads to increased miR21 and decreased vascular endothelial growth factor (VEGF)
3. BRAF V600E mutation leads to an increase in integrin heterodimerization, which leads to decrease in VEGF expression and increase in MAPK signaling
The assay offers a powerful technique for multiomic analysis and accurate interrogation of complex tissues to obtain insights into novel biomarkers and therapeutic targets. In addition, the method can contextualize the biodistribution of cell and gene therapies with potential protein markers for cell types and further interrogate cell-cell/protein-protein interactions.

P0975

Overcoming traditional limitations: GenScript's advanced mRNA Applied Vector and Strain for pertaining poly(A) integrity and efficient plasmid production

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mRNA technology has rapidly emerged as an important tool in the biomedical field for vaccine development and gene therapy, demonstrating significant advantages in accelerating drug development. The primary technical route for mRNA production is in vitro transcription (IVT), and the quality of the DNA template containing the poly(A) sequence is a key factor in determining of the quality of the final product, especially the integrity and uniformity of poly(A) tails. However, poly(A) sequence in the DNA template is prone to deletion during the production process, resulting in reduction of the DNA template quality, thereby affecting the expression efficiency and function of the mRNA product. Additionally, instability of poly(A) tails would lead to insufficient DNA yield, significantly prolong manufacturing process and cost of raw mRNA materials in therapeutics development. Therefore, there is an increasingly urgent demand in the market for more stable and high-yield mRNA application vectors and corresponding production strains. To solve those problems, we analyst, dissected, re-assembled and further modified commercial vectors, achieved the proprietary mRNA template vector with significant improved performance in poly(A) tails' integrity and stability, as well as raised production quantity. Our patented vectors, GS-mK and GS-CMV, have shown substantial improvements compared to their pre-evolution versions. The cloning success rate of the poly(A) sequence increased from 31.2% to 68.0%(GS-mK compared to pUC57), and the plasmid yield increased by 6.5 times (GS-CMV compared to pVAX1). Built-in poly(A) tails existed in those patented vectors, which can be utilized

for the efficient production of linear IVT templates. In addition, based on the mechanism of poly(A) sequence deficiency, we have developed particular strains targeting mRNA template plasmid production which further boosts the stability of the poly(A) sequence and facilitates plasmid yield. Compared to frequently used commercial strains, Genscript proprietary strains increased poly(A) cloning success rate from 23.1% to 46.6%, and the fermentation yield more than two times. It is necessary to mention that after combined using of our patented vector and stains at the same time, a superior synergistic effect has been observed. No poly(A) sequence deficiency was detected in 120A-length vectors after 10 passages incubation in 37°C.In contrast, significant poly(A) sequence deletion was observed in the corresponding commercial vector and strain as early as the 3rd generation, with more than 50% plasmids involved in half-length of poly(A) tails deficiency by the 5th generation. In conclusion, by applying GenScript mRNA vectors and strains, we can significantly improve the quality and integrity of DNA templates in mRNA production, thereby providing a solid foundation for the further development of mRNA technology.

P0977

New Biocompatible Nanohydrogels of Predefined Sizes for Complexing Nucleic Acids

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The advent of protein expression using m-RNA applied lately for treating the COVID pandemic, and gene editing using CRISPR/Cas9 technology for introducing DNA sequences at a specific site in the genome, are milestones for the urgent need of developing new nucleic acid delivery systems with improved delivery properties especially for in vivo applications. We have designed, synthesized, and characterized novel cross-linked monodispersed nanohydrogels (NHG's) with well-defined sizes ranging between 50–400 nm. The synthesis exploits the formation of self-assemblies generated upon heating a thermo-responsive mixture of monomers. Self-assemblies are formed and polymerized at high temperatures resulting in NHGs with sizes that are predetermined by the sizes of the intermediate self-assemblies. The obtained NHGs were chemically reduced to lead particles with highly positive zeta potential and low cell toxicity. The NHGs form complexes with DNA, and at optimal charge ratio the size of the complexes is concomitant with the size of the NHG's. Thus, the DNA is fully embedded inside the NHGs. The new NHGs and their DNA complexes are devoid of cell toxicity which together with their tuned sizes, make them potential tools for gene delivery and foreign protein expression.

P0978

Efficient, scalable manufacturing of virus-like particles for the delivery of gene editor ribonucleoproteins using a cGMP-compliant electroporation platform

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Genome editing tools such as CRISPR-Cas9 nucleases, base editors, and prime editors hold tremendous promise for treating human diseases by being able to specifically modify a targeted region of the DNA genome. However, there are limited *in vivo* delivery options that are safe, efficient, and transient. Moreover, viral vectors such as AAV are hampered by limited cargo size capacity. Virus-like particles (VLPs) offer a solution to these problems. VLPs are derived from retroviral structural proteins, which can be engineered to specifically package a cargo of interest. They are safer than traditional viral vectors because they lack a viral genome but can utilize the traditional virus delivery machinery to target and enter cells. Recently, VLPs have been reported to efficiently package base editors and prime editors for delivery into mice at therapeutically relevant levels. To realize the potential of VLPs for *in vivo* delivery, alternative methods to scale up their manufacturing will be critical for future clinical applications. Here, we utilized the MaxCyte® ExPERT GTx™, a cGMP-compliant electroporation instrument, to manufacture VLPs—packaged with CRISPR-Cas9 or base editor (BE) ribonucleoproteins (RNPs) for genome editing in target cells — human immortalized HEK293T cells and primary immune cells. We found that electroporation consistently produced significantly higher yields of functional VLPs compared to a commercially available transfection reagent, with greater than 10-fold improvement in p30 titer when using an optimized electroporation protocol. We also demonstrate effective genome editing at the B2M locus, with both CRISPR-Cas9 and BE RNP-packaged VLPs. Furthermore, the production of VLPs using electroporation exhibited favorable production kinetics compared to other transfection methods, enabling a shorter VLP manufacturing process. Finally, we demonstrated the scalability of VLP production across a 15-fold volume range with minimal re-optimization. In summary, our results show that electroporation is a viable means for consistent, efficient, and scalable manufacturing of VLPs for gene-editing applications and has high promise to address needs for future clinical and commercial VLP manufacturing.

P0979

ThecaFlex DRx™ System: Implantable intrathecal catheter and subcutaneous port platform for repeated intrathecal delivery of genetic therapies to the central nervous system

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Many genetic therapies, including antisense oligonucleotides (ASOs), RNA-based therapies, and biologics for the treatment of CNS diseases require repeated life-long intrathecal (IT) dosing, typically done as a standard lumbar puncture (LP) using a needle to access the lumbar IT space. LP can be challenging for those with complex spinal anatomy or requiring repeat anesthesia and radiation exposures, and complications such as post-LP headache may occur. Given these challenges, some patients may benefit from an alternative mode of therapeutic delivery.

To date, there are no US Federal Drug Administration (FDA) approved or cleared IT catheter and subcutaneous port systems indicated to provide long-term intrathecal access for repeat bolus intrathecal administration of drugs. The ThecaFlex DRx™ System, which is part of Alcyone's Falcon™ Intra-CSF Drug & Delivery Development Platform, is designed to address this high

unmet medical need. This system has undergone extensive pre-clinical testing including design verification, *in vitro* functional testing, biocompatibility and extractables, drug-device compatibility, in vivo studies in ovine models demonstrating safety and functionality, and extensive human factors usability evaluations with clinicians. The design allows for both aspiration of CSF and infusion of therapeutics and is intended to reduce rate of failures due to kinking, breakage, occlusion, migration or catheter-port disconnection, when compared to available implanted systems used off-label.

The ThecaFlex DRx™ System is an investigational device that has received Breakthrough Device Designation from the FDA and has gained CE Mark Approval in Europe. The safety and performance of the system is currently being evaluated with an ASO (nusinersen) in people with spinal muscular atrophy in an IDE clinical trial (NCT05866419). The IDE trial will enroll up to 90 patients and is being conducted in two stages. The first stage, consisting of 10 patients enrolled, implanted, and followed for 30 days, is complete. Preliminary results demonstrate that ThecaFlex is performing as intended, with no device-related adverse events observed during the first stage of the IDE trial. Based on review of the data from the first stage, the FDA has provided approval to continue enrollment of an additional 80 patients. The IDE trial may now enroll up to 90 patients from up to 30 clinical sites across the U.S. and Europe.

Potential clinical benefits may include:

- Mitigating the challenges associated with repeat LP dosing, including potential reduction in the need for anesthesia and/or radiation for therapy, infusion, and monitoring, especially for patients with complex spinal anatomy.
- Potentially enabling routine subcutaneous delivery of ASO or other genetic therapies to the cerebrospinal fluid and provide an easier approach to CSF sampling.

Combined, these benefits could improve the patient treatment experience of long term, repeat intrathecal administration of therapies to the CNS. Importantly, the ThecaFlex DRx™ IT catheter and port system is applicable to a broad patient population diagnosed with neurological disorders that require repeat IT dosing of therapeutic agents. Biogen has reviewed this abstract. The authors had full editorial control of final content.

P0980

A straightforward solution for transfection complexes size kinetics follow-up, the Videodrop allows to better characterize, optimize and control the transfection step

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In bioproduction up stream processes (USP), the transfection step is essential as it has a direct impact on the final bioproduct functionality and on the yield of the overall process. Mastering the efficiency and reproducibility of the transfection is critical to control bioproduction cost and quality.

Among the existing approaches, the chemical transfection using polymeric genes carriers is widely used by the industry. Plasmids coding for the bioproduct are mixed with polymers to form a

complex that enters within the cell through endocytosis. This complexation is a dynamic process implying that complexes size increases with time. The literature shows that transfection efficiency depends on the complex size. Consequently, being able to monitor this size kinetics is essential to have access to a key parameter for the transfection step process development, scale up and in process control.

In this study, we introduce and showcase how the Videodrop, a fast & user-friendly nanoparticle analysis instrument, and its new size kinetics algorithms, allow to follow in real time the size evolution of transfection complexes from 80 nm to 2um. One droplet of few microliters of transfection solution allows to record a live kinetics. Several typical transfection molecules and conditions were tested. The repeatability and accuracy were also assessed.

These results indicate that the Videodrop is a new suitable instrument, adapted to USP team constraints and reality, to characterize the size kinetics of transfection complexes. It gives a new insight into the optimization and control of the transfection step

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P0981

Monitoring adenoviral gene therapy safety

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Gene therapies utilize both integrating and non-integrating viral vectors. Adenoviral vectors are non-integrating vectors that form episomes within host's cells and are used in gene therapy for stable expression of therapeutic gene(s). Adenoviral vectors (Ad) are one of the first gene therapy vectors used in the clinic and are derived from wildtype adenoviruses. They are widely used in vaccines and gene therapy including oncolytic therapies. One of the commonly used Ad vectors is based on wild-type adenovirus type C5, (Ad5), widely present in the environment. The Ad5 vector has been engineered to be replication-deficient, conditionally replicating or replicating vectors for killing cancer cells, called oncolytic therapies. Humans develop immunity against wildtype Ad5 and are naturally shed from the body through secretions without any harm to the host. Thus, any potential therapy with these Ad5 vectors needs monitoring of the therapy for shedding through body secretions. There is also a hypothetical chance that replication-deficient Ad5 vectors administered to a subject can recombine with wild type cousins present in the subject and become replication competent adenoviruses (RCA). This needs sensitive assays that can determine shed vector particles and any potential RCA. For the above mentioned reasons, we have developed qPCR assay each for detecting shedding and RCA in blood, plasma and nasal swab samples. Since qPCR cannot distinguish between infectious and non-infectious vector particles, a sensitive infectivity assay to detect the vector infectivity has also been developed. If RCA is present, we have developed an unbiased sequencing and analysis pipeline based on Oxford Nanopore sequencing to precisely identify the sequence within the recombined Ad5, that rendered it replication competent. Thus, we have comprehensive set of assays that sensitively detect any shedding from Ad5 vectors and determine if the shed vectors are replication competent and infectious or not. These assays are essential in any clinical trial to monitor shedding and determine if the shed vector particles are not harmful to ensure appropriate safety of Ad5 gene therapy including oncolytic or therapies.

P0982

High-Throughput Digital PCR Method for CRISPR HDR Knock-In Quantification Using a Unique Linkage Assay

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Gene therapy holds transformative potential across medical applications, including cancer treatment, genetic disorders, and regenerative medicine. Accurate quantification and validation of gene edits are crucial for these therapies' success. This study introduces a novel high-throughput digital PCR (dPCR) method to verify and quantify CRISPR HDR (homology-directed repair) knock-in events using a TaqMan probe-based linkage assay, providing a versatile tool for multiple gene therapy applications.

CRISPR HDR knock-in requires a donor template with homology arms flanking the target site for precise integration. Standard dPCR methods are inadequate for detecting locus-specific integration, typically measuring only transgene presence without verifying correct insertion. Some assays have been proposed with primer pairs spanning from the target locus across homology arms to the transgene, but due to the long amplicon size these assays have an increased PCR failure rate with limited sensitivity.

Our method overcomes these limitations with a linkage assay that combines three distinct assays into a single reaction, enabling quantification of transgene copy number at the correct target site. Our linkage assay is a triplex assay comprising three assays: the target locus assay (located 400 to 800 bp from the cut site, beyond the homology arms region), the transgene assay, and a reference assay for cell count detection. For simultaneous amplification and quantification, a multiplex strategy using three sets of forward and reverse primers and three fluorophore labeled probes is employed: HEX for the endogenous control, FAM for the transgene, and ROX for the target locus.

The linkage assay relies on the principle of random and non-random distribution of target sequences across partitions. Linked sequences localize to the same partitions unless separated by restriction digestion during PCR preparation. We used an endonuclease to digest the human genome before the first PCR cycle and chose enzyme that does not cut within or between linked assays. Correct knock-ins result in both targets (transgene and target locus) being present in a single partition, increasing double-positive partitions compared to unlinked samples where random distribution occurs. This provides a robust statistical framework to calculate non-random double-positive partitions, ensuring precise quantification of correct knock-in events.

We validated our approach in an in vitro cancer gene therapy setting where cytokine-coding transgenes were integrated in neuroblastoma cell lines to enhance immune cell infiltration and improve immunotherapy efficacy. Our dPCR method demonstrated high sensitivity and specificity in quantifying CRISPR knock-in events and could detect and quantify correct integration as early as 24 hours after knock-in.

In conclusion, the high-throughput digital PCR method developed in this study represents a versatile tool for quantifying CRISPR HDR knock-ins. Its applications span multiple fields of gene

therapy, from cancer treatment to genetic disorder correction and regenerative medicine. By accurately measuring genetic modifications, this method facilitates the development of effective and safe gene therapies, ultimately contributing to improved patient outcomes across various medical disciplines.

P0983

Unveiling the potential of menstrual blood-derived stromal cell (MenSC) secretome as a cell-free therapy for ovarian cancer

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Ovarian cancer is often diagnosed at an advanced stage and treatment options are limited, underscoring the need for innovative treatment strategies. The OVCAR8 cell line, derived from a high-grade serous ovarian carcinoma, serves as a model to understand the pathophysiology of the disease and explore new therapeutic strategies. In this context, the secretome released by menstrual blood-derived mesenchymal stem cells (S-MenSCs), either in their basal state (S-bMenSCs) or after priming with IFNγ and TNFα (S-pMenSCs), offer a promising approach to treatment. This study aims to assess the in vitro efficacy of these treatments in ovarian cancer, focusing on their effects on cell proliferation, cell cycle regulation, and cell viability. For this, MenSCs (n=5), previously isolated, were cultured in complete DMEM medium (10% fetal bovine serum (FBS) and 1% penicillin-streptomycin) for 72h for basal condition (bMenSCs) or in DMEM supplemented with IFNg and TNFa at 100 ug/ml for primed condition (pMenSCs). Subsequently, the medium was replaced with serum-free medium for 48h. In both cases, the resulting conditioned medium was collected, centrifuged to remove cell debris, and ultrafiltered (3kDa) to obtain the secretomes. OVCAR8 cells were treated with S-bMenSCs or S-pMenSCs at 100 µg/ml of concentration and analyzed at 0, 3, and 6 days. As a control, OVCAR8 cells were cultured in complete DMEM medium. Cell proliferation and cytotoxicity were evaluated using the Cell Counting Kit-8 (CCK8) assay. For cell cycle analysis, cells were harvested, fixed in 70% ethanol overnight at 4°C, stained with propidium iodide (PI), and analyzed using flow cytometry. Cell viability was assessed using a live/dead cell viability assay, and live and dead cell counts were quantified using ImageJ. Statistical significance was determined using GraphPad Prism software. $p < 0.05$ was considered statistically significant. As results, CCK8 assay demonstrated a significant reduction in cell proliferation following treatment with both secretomes. On day 3, proliferation was reduced by 5.5% and 20.9% with S-bMenSCs and S-pMenSCs treatments respectively, while on day 6, reductions of 15.6% and 34.6% were observed, indicating a progressive inhibition of cell growth over time. Cell cycle analyses revealed significant alterations in cell cycle phases upon treatment with MenSC secretomes. The S phase of the cell cycle exhibited a notable decrease of 33.3% and 56.8% with treatments using S-bMenSCs and S-pMenSCs respectively, while the G0/G1 phase showed a corresponding increase of 12.4% and 27.2%. Further assessment of viability showed no discernible differences between treatment conditions; however, a higher proportion of dead cells were observed with S-pMenSCs treatment, suggesting a potential cytotoxic effect. In conclusion, the findings of this study suggest that secretome released by MenSCs, particularly when primed, exerts significant anti-proliferative effects on ovarian cancer cells, potentially through modulation of cell cycle progression and induction of cytotoxicity. These

results provide valuable insights into the therapeutic potential of S-pMenSCs as a novel cell-free therapy for ovarian cancer, warranting further investigation and clinical translation.

P0984

ASO-mediated inhibition of MyD88L265P-dependent signaling as a therapeutic approach for Waldenström's macroglobulinemia

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MyD88 is an adaptor protein involved in the signaling pathways of Toll-like receptors (TLRs) and the Interleukin-1 receptor family, which are crucial for innate immune responses. Its somatic gain-of-function mutations, in particular MyD88 Leu265Pro ($MyD88^{L265P}$), are implicated in lymphoid malignancies such as Waldenstrom's macroglobulinemia (WM), where more than 90% of patients have been shown to carry the mutation. $MyD88^{L265P}$ promotes lymphoma cell survival by upregulating the expression of Bcl-xL and activating Burton tyrosine kinase (BTK), while also having an effect on the bone marrow microenvironment by transmission of $MyD88^{L265P}$ via extracellular vesicles (EVs) to propagate inflammation. Our research explores a gene therapy strategy using antisense oligonucleotides (ASOs) to induce exon skipping of MyD88, resulting in a dominant negative isoform (MyD88 short, $MyD88_S$) that inhibits $MyD88^{L265P}$ signaling. In vitro, our ASOs successfully altered exon splicing to produce the short isoform and nearly completely inhibited IL-1β signaling. Experiments on the malignant B-cell line MWCL-1, which harbors the L265P mutation, demonstrated significant cytokine secretion inhibition and apoptosis induction. Notably, ASOs outperformed current small molecule therapies, including ibrutinib and venetoclax, in these aspects. We also showed efficient in vivo ASO delivery using lipid nanoparticles (LNPs) conjugated with anti-CD38 antibodies, achieving targeted localization into the bone marrow, spleen, and lymph nodes. Our findings highlight the therapeutic potential of ASOs for WM treatment.

P0985

Engineered epigenetic silencers repress glioblastoma growth

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Current therapies remain unsatisfactory in preventing the recurrence of glioblastoma multiforme (GBM), which leads to poor patient survival. Major evidence indicates that cancer cells with tumor-initiating potential infiltrate the parenchymal tissue and become resistant to adjuvant treatments, thereby supporting the recurrence of the disease.

A treatment based on the administration of epigenetic editors *in vivo*, e.g. post-surgery brain, can extinguish the GBM resistant cells and minimize the risk of tumor reappearance, without risk for the surrounding (healthy) cells.

We aim to define the role of a new class of epigenetic silencers in coordinating the anti-tumor activity (1), their effective functionality in preventing tumor growth (2), and their safety (3). By rational engineering of oncogenic transcription factors through the rational assembling with domains from chromatin and transcriptional epigenetic repressors, we generated a family of synthetic epigenetic silencer factors (ESFs). The modified transcription factors include SOX2 and TEAD1 which have of the key in GBM. Through the addition of the KRAB and DNA methyltransferase 3A/L catalytic domains, we generated synthetic repressors named SOX2 epigenetic silencer (SES) and TEAD1 epigenetic Silencer (TES). Both ESFs were tested in *in vitro* and *in vivo* models of GBM as well as their molecular activity, transcriptional and anti-tumoral outcome.

SES and TES inhibit both glioma cell lines and patient-derived cancer stem cells *in vitro* and *in vivo*. Their expression, through local viral delivery in mouse xenografts, induces strong regression of human tumors and survival rescue. SES produces a significant silencing of a large fraction of the SOX2 transcriptional network, achieving high levels of efficacy in repressing aggressive brain tumors. TES induced the selective and robust transcriptional silencing of the YAP/TAZ validated targets, through epigenetic inhibition as assessed by RNA-seq. Conversely, they are not harmful to neurons and glia, representing e relevant option of GBM therapy.

Collectively, this rational protein engineering approach produced a significant silencing of a large part of the tumorigenic transcriptional network, achieving high levels of efficacy and safety for the treatment of aggressive brain tumors. Given its wide applicability to other oncogenic TFs and the efficiency of targeting cancer cells by viral transduction, this approach offers an innovative strategy to build antitumor molecular tools effective against glioblastoma and other deadly cancers.

P0986

Insights into endometriosis pathogenesis: understanding the impact of proinflammatory priming on menstrual blood-derived mesenchymal stromal cells (MenSCs)

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While retrograde menstruation remains the predominant theory, the pathogenesis of endometriosis is not fully understood. Consequently, there is a growing interest in elucidating the role of menstrual blood-derived mesenchymal stromal cells (MenSCs) in the genesis of this complex condition. Acknowledging the inflammatory nature of endometriosis, this study aims to examine in vitro the impact of proinflammatory priming with interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα) on the potential pathogenesis capacity of MenSCs in endometriosis. For this, MenSCs were isolated, cultured, and subjected to proinflammatory priming. Transcriptomic analysis was used to assess the impact of inflammatory pretreatment on gene and miRNA expression patterns related to endometriosis. Our comparative study integrated

transcriptomic data with genes differentially expressed (DEGs) in tissues of endometriosis patients obtained from Malacards (the Human Disease Database). We also included the DEGs in endometriotic MenSCs (eMenSCs) compared to MenSCs described in the literature for the analysis. Additionally, the miRNA profile derived from the transcriptomic data was contrasted with miRNAs associated with endometriosis from the Human microRNA Disease Database (HMDD) v4.0. Notably, among the 73 endometriosis DEGs, only IDO1, CLU, and KLF2 were differentially expressed with proinflammatory priming compared to basal MenSCs gene expression. There was no overlap with DEG eMenSCs. Regarding miRNAs, 14 out of 40 differentially expressed with priming were associated with endometriosis, although only half exhibited the same trend. In conclusion, these findings suggest that the pathogenesis of endometriosis likely involves multifaceted mechanisms beyond a simple inflammatory response in MenSCs. Further research into the pathogenesis of endometriosis is essential to unveil its complex molecular landscape and identify potential therapeutic targets.

P0987

Design of a retinoid-responsive toxin gene therapy approach for neuroblastoma treatment

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Retinoic acid (RA) has been shown to improve clinical outcomes for neuroblastoma (NB) patients due to its ability to induce differentiation in tumour cells. However, its use has been limited by toxicity and tumour-acquired resistance after prolonged treatment, following which patients relapse and this is often fatal. To increase the efficacy of RA, we aim to design a “toxin gene” therapeutic that is highly sensitive to transcriptional activation by RA. When tumour cells take up the DNA-based agent, they will be bathed in exogenous RA and be rapidly killed. This will effectively re-purpose RA, converting the differentiation response from a cytostatic to a cytotoxic response. We have linked the toxin gene diphtheria toxin A (DTA) to RA-responsive promoters in plasmids and tested their cytotoxic capabilities under a range of RA concentrations in cultured cell lines. MicroRNA-response-elements (MREs) were also cloned into the plasmids to minimise expression in off-target tissues, primarily the liver. We have then used liposomes coated with NB-targeting peptides to assess delivery of our plasmids to cells in culture. The RA-driven DTA vector, after transfection into NB cells, is highly sensitive to RA, requiring minimal exogenous RA (<100 nM) for maximal cytotoxic activity. Compared to RA alone, the vector shows greater toxicity at all concentrations of RA, shown by the complete loss of positively transfected NB cells following RA treatment; this is seen even in the more RA-resistant SK-N-AS cell line. Liver-specific MREs knocked down transcription by 98% in the hepatoma-derived Huh7 cell line, and also reduced cytotoxicity in this line; however, decreased activity was seen in some NB cells. The liposome-based nanocomplexes also delivered sufficient levels of DTA-expression plasmid *in vitro* to trigger efficient cell death in a range of NB-derived tumour cell lines. We have developed an inducible gene therapy approach that is able to achieve greater tumour cell cytotoxicity in culture than RA alone. Liver-specific MREs provided some protection against toxicity in liver cells, while cytotoxicity in NB lines was sufficiently maintained. Next steps are to improve the tumour-selectivity of the toxin gene and to carry out proof of principle testing *in vivo* using our targeted deliver system. If successful, this novel approach could be developed as a combination treatment alongside RA in patients, as a route to more effectively remove residual tumour cells.

P0988

Embedding viral capsids into aggregated metallic nanoparticles improves adenovirus entry in various human cancer cells

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Delivery of macromolecules or nano-objects by electrotransfer is an attractive procedure in many fields of biotechnology and gradually in therapeutics. When using low amplitude/long lasting electric pulses to facilitate adenovirus delivery into cancer cell lines, we previously showed an enhanced virus penetration, not directly resulting from pulse application nor to membrane modifications, but rather due to viral capsid interactions with aggregated metallic nanoparticles, released from energized electrodes. In this work, we have quantified the viral and metallic particles forming these complexes by nanoparticle tracking and Inductively Coupled Plasma-Atomic Emission Spectroscopy, respectively. The intimate structure of these complexes and their cell penetration were investigated by transmission electronic microscopy and confocal microscopy. The new properties conferred on viral capsids by aggregated metallic nanoparticles were further studied. We characterized their contribution to virus stability and to penetration of embedded viral capsids into cell lines derived from several human cancers, including some Head and Neck carcinoma cell lines poorly infected with adenovirus vectors. Using a panel of endocytosis inhibitors, we found that macropinocytosis is the main penetration pathway for the viral capsids associated with the metallic aggregates. The previous properties could be useful to reduce the virus doses required for tumor treatments with oncolytic adenoviruses and/or adenovirus-derived vectors expressing immunomodulatory protein(s). The modification of the virus stability and cell entry process might also modulate the quality and intensity of the immune response induced against the viral vector.

P0989

Polypurine Reverse Hoogsteen hairpins as a silencing gene tool against estrogen receptor positive breast cancer cells

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In this work we used Polypurine Reverse Hogsteen (PPRH) hairpins as a silencing tool to inhibit the expression of the Estrogen Receptor alpha (ERα), a hormone receptor that is overexpressed in 70% of breast tumors. Two PPRHs against the *ESR1* gene, HpER3 and HpER10, were designed and transfected in the ERα-positive breast cancer cell lines ZR-75-1, MCF-7 or T47D. In all cases, both PPRHs successfully decreased cell viability and ESR1 expression levels. The effectiveness of the PPRH silencing technology was also evaluated *in vivo*. ZR-75-1 cells were transfected with 250nM of each PPRH and inoculated on the Chicken Chorioallantoic Membrane (CAM) model for one week. Tumors were weighted, and their histological profile was analyzed using hematoxylin-eosin staining. Although weight differences were not significant, the tumors originated from PPRH-transfected cells exhibited a different profile from the controls, with fewer tumorigenic cells and less ductal aggregates. Immunofluorescence analyses of the sectioned tumors were also performed to compare the expression of both ERα and Ki67, a proliferation marker. ERα mRNA and protein expression levels from pooled tumor samples of the different conditions were assessed, revealing a decrease in ERαlevels in HpER3 and HpER10 treated tumors. Based on these results, we can conclude that the designed PPRHs effectively decrease the expression of ERαboth at the mRNA and protein levels, *in vitro* and *in vivo*, thereby reducing the presence of tumorigenic cells. This study suggests that the PPRH technology may be a potential alternative treatment to chemotherapy or hormone antagonists, by directly targeting ERα expression.

P0990

TROCEPT- an oncolytic virus platform engineered for $\alpha_v\beta6$ integrin targeted tumor-localized expression of an immune checkpoint inhibitor following intravenous delivery

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TROCEPT is a novel tumor-selective replicating, oncolytic adenovirus type 5 (Ad5) rationally engineered to remove all natural tissue tropisms. This engineering addresses the main limitation of other viral therapies which infect normal tissues and are rapidly removed by the liver and are therefore limited to local delivery. TROCEPT has been further engineered to specifically bind to αvβ6 integrin which is expressed at high frequency in the majority of epithelial cancers, so that following intravenous delivery the virus targets tumor tissue where it replicates and amplifies. In addition, the TROCEPT platform encodes transgenes which enables in-tumor production of powerful therapeutic drugs. The lead programme, TROCEPT-01, encodes a fully human, full length immune checkpoint inhibitor (ICI) antibody. TROCEPT-01 is currently undergoing IND-enabling studies and is expected to enter First in Human studies in 2024 in multiple solid tumor indications.

Proof of concept studies, both *in vitro* and *in vivo*, to demonstrate TROCEPT-01's tumor selectivity and toxicity profile have been performed.

In vitro experiments demonstrate that viral replication, transgene expression and oncolytic cell death following infection with TROCEPT-01 is highly selective for αvβ6 integrin positive tumor cells compared to a panel of normal human primary cells. Intravenous delivery in *in vivo* SKOV3 ovarian human tumor xenograft mouse models demonstrate virus delivery, replication, and transgene expression in the tumor. In comparison to the parental Ad5 virus, the engineering of TROCEPT-01 leads to more viral delivery to the tumour, less delivery to normal tissue including liver, reduced markers of liver toxicity and reduced inflammatory cytokine release, all of which validate the tumour selectivity and safety profile of the virus.

We have demonstrated that TROCEPT engineering enables highly selective tumor targeting and significant tumor exposure following intravenous delivery. TROCEPT-01's in-tumor selective expression of ICIs enables high local drug concentration in the tumor, potentially reducing systemic toxicity and increasing efficacy. TROCEPT-01 is currently undergoing IND-enabling studies and is expected to enter First in Human studies in 2024 in multiple solid tumor indications.

P0991

Programmable genome disruption enables selective elimination of cancer cells using a novel variant of Cas12a2 nuclease

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The utilization of the prokaryotic immune defence system CRISPR-Cas for mammalian applications has significantly boosted genome engineering for basic research and medical applications in the last decade and has promoted the mining for new Cas variants with advantageous editing properties. Our metagenomics screen revealed a novel Cas12a2 nuclease variant, termed G-dase E, with an unexpected mode of action. G-dase E forms a ribonucleoprotein (RNP) complex containing both the Cas12a2 enzyme and a specific guide RNA (gRNA) that identifies and binds a target RNA molecule. Following gRNA-mediated recognition of the target RNA (e.g. mRNA), the collateral activity of G-dase E is activated, catalysing non-specific cleavage of RNA and DNA, which ultimately leads to cell death. The programmable collateral activity of the G-dase E nuclease opens up a new possibility of cancer treatment by targeting and eliminating cancer cells based on an oncogenic RNA marker. In cytotoxicity studies, we were able to show that the use of G-dase E RNPs targeting the clinically relevant oncogenes HPV18-E6/E7 in HeLa and HPV16-E6/E7 in CaSki and SiHa cervical carcinoma cells leads to selective, targeted cell depletion, while oncogene-negative cells show no effect on cell viability. Although the use of CRISPR-Cas RNPs is very promising and offers numerous advantages such as rapid onset, low toxicity, fewer immune responses and minimizing risk of foreign DNA integration, RNPs have mainly been used in ex vivo approaches so far while in vivo RNP delivery is still in its infancy. In contrast, nucleic acid-based in vivo delivery methods such as mRNA-LNP and viral vectors are much more advanced in therapeutic development. In cell culture studies, we were able to show that G-dase E can be delivered into human cells as mRNA combined with synthetic gRNA. Cytotoxicity assays in human cells showed that mRNA delivery of G-dase E induces selective, RNA marker-specific, elimination of target cells comparable to G-dase E RNPs. The possibility to apply different delivery forms for G-dase E now paves the way into preclinical in vivo cancer models to assess the potential of the novel Cas12a2 variant for targeted tumor depletion. Overall,

we have shown that the programmable G-dase E can be delivered as RNP and mRNA to induce selective cell elimination upon recognition of user-defined marker RNAs, providing an innovative and flexible strategy for targeted cancer therapies.

P0992

Microneedle patch encapsulating Peptide-coated Conditionally Replicating Adenoviruses (PeptiCRAd) as transdermal tumor vaccine for melanoma

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Cancer immunotherapy stands at the forefront of modern oncology, empowering the immune system to recognize and eradicate malignant cells. Recent clinical trials have illuminated the potential of tumor vaccines, yet their broad application is frequently impeded by challenges in effective antigen delivery and presentation. Microneedles (MNs), characterized by their minimal invasiveness and efficient penetration of the skin's barrier, have emerged as a promising solution. These devices not only deliver antigens directly to the skin's immunologically active layers but are also proven to effectively encapsulate and release both traditional and novel vaccine components, thereby enhancing immune responses. For this reason our team formulated a microneedle patch encapsulating Peptide-coated Conditionally Replicating Adenoviruses (PeptiCRAd), marking a strategic evolution in oncolytic virus-based cancer vaccines. PeptiCRAd leverages the robust immunogenicity of adenoviruses, refining the targeted immune response by complexing the virus with tumor-associated antigens (TAAs). This approach not only enhances direct delivery and presentation mechanisms but also tailors the immune system's focus, shifting it from viral components to tumor-specific antigens. Such synergy boosts the efficacy of CD8+ T-cell mediated attacks on tumor cells, crucial for surmounting the limitations of traditional treatments that often fail to differentiate between antiviral and antitumor responses. The integration of PeptiCRAd with MN technology involves a specialized blend of polyvinylpyrrolidone (PVP) and sucrose to optimize the vaccine's delivery efficiency. This combination is pivotal in maintaining the structural integrity and viability of MNs, with sucrose acting as a lyoprotectant during the fabrication process, crucial for preserving the virus's infectivity once administered into the dermal microenvironment.

In vivo experiments demonstrated that MN-delivered PeptiCRAd effectively activated immune responses, comparable to subcutaneous injections. Mice treated with PeptiCRAd MNs showed complete tumor rejection, enhanced dendritic cell populations, increased effector memory cells, and CD8 T-cells expressing IFN-gamma. ELISA and ELISpot assays revealed robust antibody and T-cell responses against adenovirus and SIINFEKL peptide, respectively, with MN delivery showing superior T-cell activation.The integration of PeptiCRAd with MN technology also highlighted the importance of dendritic cell activation. Flow cytometry analysis revealed significantly elevated CD86 expression in cells treated with PeptiCRAd MNs. Furthermore, peptide presentation on MHC class I molecules was robust in PeptiCRAd and Peptide MN groups, critical for effective T-cell recognition.

This study confirms the practicality and adaptability of MNs and PeptiCRAd as effective options in cancer immunotherapy. The use of sucrose in MN formulation preserves viral infectivity, while the combination of adenovirus and tumor antigens in PeptiCRAd enhances immune responses.

MN delivery not only improves patient compliance due to its minimally invasive nature but also ensures efficient antigen delivery and presentation, crucial for robust immune activation. Future research should focus on overcoming scalability and stability challenges to fully harness the potential of MN technology in oncology.

P0993

In vitro and in vivo characterization of novel “armed” oncolytic viruses derived from different adenovirus species for breast cancer therapy

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Oncolytic adenoviruses (OAds) are promising candidates for cancer therapy. Current studies and clinical trials focus on human adenovirus type 5 (Ad5), but robust therapeutic effects were hindered due to two major factors: low expression levels of the main Ad5 coxsackie and adenovirus receptor (CAR) on tumor cells and the high seroprevalence of Ad5. Here we aimed at exploring alternative Ad types with low seroprevalence and different receptor usage as an attractive source for OAd development. Based on our previous work, comparing more than 20 Ad types regarding their ability to transduce different human breast cancer cell lines, two Ad types were selected. Ad34 and Ad35, utilizing CD46 as receptor, and especially Ad34, displaying a low seroprevalence, were transformed into OAds. Here, the original E1A transcription unit promoter, responsible for adenovirus genome replication, was replaced by the tumor-specific human telomerase reverse transcriptase promoter (hTERT). To maintain Ad5 derived strong lysis effects and to “arm” the non-Ad5 Ad types, the adenovirus death protein (ADP) from Ad5 was inserted into the E3gp19k region of Ad34 and Ad35 genomes. As a control and for direct comparison Ad5-hTERT was generated.

The constructed OAds Ad5-, Ad34-, Ad35-hTERT, Ad35-hTERT-TATA, Ad34- and Ad35-hTERT-ADP were analyzed for infectivity, lysis and cell viability in four breast cancer cell lines (Hs578T and MDA-MB-231 (both triple negative, TNBC), MCF-7 and SKBr3) and in A549 cells. For titration and characterization of all generated viruses, TCID50 assays to determine plaque forming were performed in all analyzed cell lines. Oncolysis and viability assays performed in all breast cancer cell lines revealed that both “armed” ADP-OAds resulted in reduced cell viability and therefore superior cell killing effects compared to the control vector Ad5-hTERT. Next we performed neutralization assays with anti-human-Ad5 serum using dilutions from 1/16,000 to 1/125. Results revealed neutralization of Ad5 but not of Ad34 and Ad35 demonstrating that both Ad34 and Ad35 vectors are interesting candidates for *in vivo* applications. *In vivo* experiments in nu/nu NMR1/FoxN1 mice bearing subcutaneous A549 xenografts showed a highly significant decrease in tumor growth with ADP-OAds compared to hTERT-only-OAds 27 days post-engraftment and 15 days after intratumoral application of 1x10e9 viral particles (vp). To examine if the OAds can reach the tumor site when injected intravenously, three additional OAds based on Ad5, Ad34 and Ad35 were generated encoding firefly luciferase (fLuc) driven by the SV40 promoter instead of the E3gp19K transcription unit. Biodistribution experiments showed high replication-activity in the tumors after intravenous injection of 1x10e9 vp as quantified by qPCR. Moreover, viral DNA for Ad34 and Ad35 was detected in lymph nodes in close proximity to the tumor, pancreas and spleen. To address the safety profile of the OAds and transduction

properties in human blood cells, non-activated and activated T-cells from nine donors were infected. As an important safety profile, five days post-infection no significant lysis was observed.

P0994

Multi-omics integration for enhanced peptide discovery for oncolytic viral-based cancer vaccines in acute myeloid leukemia

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Cancer Immunotherapies have dramatically changed how cancer is treated nowadays. Advances in understanding anti-tumor immune response have provided novel strategies to target malignant cells precisely. In particular, most anti-cancer vaccines and adoptive T-cell therapies rely on accurately identifying and validating tumor-specific and tumor-associated antigens (TSAs and TAAs, respectively) displayed on the tumor surface through HLA-I molecules. Unfortunately, many antigens are poorly shared among patients and in addition, they show a significant degree of intra-tumoral heterogeneity. Furthermore, malignancy is associated with endogenous retroviruses (ERVs) reactivation upon genetic and epigenetic deregulation, a phenomenon observed in patients. Since ERVs and other transposable elements (TEs) constitute over 45% of our genome while being unexpressed in normal cells, their reactivation could provide novel immunogenic HLA-I ligands, thus increasing the patient cohort's eligibility for therapeutic cancer vaccines. Hypomethylating agents (HMAs)(e.g. Azacytidine (AZA) and Decitabine (DAC) are approved for the treatment of myelodysplastic syndrome (MS) and acute myeloid leukemia (AML); the ability to further de-repress ERVs and TEs and activate anti-viral innate immunity in cancer cells has recently been speculated to be the actual mode of action of these drugs. Despite the promising results in preclinical studies, clinical knowledge regarding the HMA-induced set of ERV ligands and their expression rate on the tumor surface is still limited. We propose a multi-omic integration pipeline for identifying and selecting ERV and TE targets upon HMA treatment for enhanced oncolytic viral-based cancer vaccines in human acute myeloid leukemia (AML).

P0995

Novel oncolytic adenovirus improves therapeutic efficacy of disseminated lung cancer

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The rising incidence of cancer and associated mortality necessitates the development of innovative treatment modalities for patients who do not respond to existing therapies. Oncolytic viruses have emerged as a promising approach in cancer therapy. We developed a novel engineered oncolytic adenovirus, AVID-317, which demonstrated a significantly improved safety profile following systemic administration due to a set of targeted mutations in the viral capsid. Systemic administration of AVID-317 at a dose of 5×10^{10} v.p. per mouse to NCr/Nude mice with

disseminated lung adenocarcinoma slowed tumor progression and significantly extended survival compared to buffer-treated control group. In the AVID-317 treated group, approximately 25% of the mice showed complete rejection of disseminated tumors, while the remaining mice exhibited significantly extended survival. Surprisingly, dose escalation of AVID-317 did not lead to improved therapeutic benefit. Instead, tumor-bearing mice treated with higher doses of AVID-317 exhibited reduced median survival.

To understand the mechanism underlying the reduction in median survival following dose escalation, we established an *in vitro* system in which single-cell suspensions prepared from the lungs of tumor-bearing mice were cultured and treated with increased doses of AVID-317. 48h post-virus infection, the cells were harvested for flow cytometry analysis, and the culture media were analyzed for released inflammatory cytokines and growth factors known to stimulate tumor growth. Our flow cytometry results confirmed that AVID-317 infection of lung cells isolated from the tumor-bearing mice eliminated transplanted human A549 tumor cells in a dose-dependent manner. However, inflammatory cytokines and tumor-stimulating growth factors in the culture media increased with escalating virus doses. Flow cytometry analysis also demonstrated that higher virus doses resulted in increased levels of the pro-tumorigenic factor PDL1 in the non-CD45+ mouse stromal cells. These results indicate that efficient tumor cell killing by the virus triggers activation of cellular components in the tumor microenvironment, which in turn produce pro-tumorigenic factors, leading to accelerated tumor growth.

To further dissect the mechanism of AVID-317-mediated tumor suppression and to design more effective therapies, we analyzed disseminated tumors from the lungs in NCr/Nude mice using single cell RNA-seq and Mass Cytometry (Cy-TOF) after virotherapy. scRNA-seq analysis showed that in a subset of mice responding poorly to AVID-317 therapy, tumor-associated CSF1R-expressing myeloid cells expressed higher levels of MHC-II, Ctsb, and Arg1. In addition, $CD3e^{+}$ T cells expressed functional exhaustion marker, PD-1, exclusively in mice that responded poorly to virotherapy. Mass Cytometry analysis with 37 metal isotope-labeled antibodies confirmed elevated expression of activation markers on myeloid cells after virotherapy. Based on these findings, we designed a combination therapy where in addition to AVID-317 administration, tumor-bearing mice were treated with anti PD-1 antibody to block immune-suppressive signaling, anti-CSF1R antibody to block CSF1R signaling, and anti-Asialo GM1 antibody to deplete anti-viral NK cells.

Overall, our studies uncover complex mechanisms that limit the therapeutic efficacy of adenovirus vectors in treating disseminated cancer. They also demonstrate that combining virotherapy with specific immunotherapeutic drugs can significantly enhance therapeutic outcomes.

P0996

Peptides identified by Adenovirus Directed EVOlution (ADEVO) result in broad-spectrum enhanced oncolytic vectors

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Directed evolution is a vector development method that consists in the generation of random vector variants libraries, followed by the selection of improved variants fitting selection criteria. Directed evolution yielded few successes in adenovirus vector (AdV) development until now, mainly due to difficulties in constructing large random libraries. To overcome this challenge, we designed novel Adenovirus Directed EVOlution (ADEVO) protocols based on random oligonucleotides insertion in chosen genomic loci using high-throughput homologous recombination. Variant libraries were constructed by random octopeptides insertions in the exposed HI loop of the fiber knob domain of human adenovirus type 5 (Ad5). Prior to selection, libraries contained up to 9.6e5 unique variants per vector library according to conservative statistical modeling based on next-generation sequencing of library aliquots. Libraries were selected by serial passaging on various cancer cell lines including pancreatic carcinoma cell lines (Panc-1, MiaPaCa-2, AsPc-1) as well as cell lines with very low permissivity for Ad5-WT, namely SkOv-3 cells (ovarian carcinoma), EAhy926 cells (endothelial cells) and A549-ΔCAR cells (lung carcinoma, deleted for Ad5's primary receptor). The goal of selection was to identify variants able to infect and lyse these cells efficiently, since such variants may represent enhanced oncolytic vector candidates. After eight rounds of selection by serial passaging of the replication-competent vectors, the most highly enriched variants were recloned and purified. Interestingly, the sets of variants selected in each cell line largely overlapped, indicating that broad-spectrum lytic variants had been selected. Furthermore, these variants did not display enhanced or more specific transduction but intensified and accelerated replication and cell lysis in all tested tumor cell lines. The selected inserts were then added to the fiber proteins of Ad5, Ad34 and Ad35-based replication-selective oncolytic vectors to increase their therapeutic index. As expected, the generated vectors displayed the same *in vitro* properties of improved oncolysis than selected library variants. Although it was not expected that short peptides insertions in the fiber protein could lead to enhanced replication and cell lysis, these features are consistent with the applied selection pressure, which was the efficient completion of full replication cycles. The mechanisms of this so far unsuspected involvement of the fiber protein in stages of the AdV replication cycle other than receptor binding are currently being investigated. ADEVO, our novel directed evolution workflow for adenoviruses, facilitates the generation of highly diverse variant libraries and the selection of improved vectors with user-friendly protocols. Besides the development of enhanced oncolytic AdVs shown here, we expect that this technology can be applied to other vector features and contribute to a wide range of clinical applications.

P0997

Oncolytic adenovirus armed with IL-12 enhances antitumor immunity in mouse models of melanoma and colorectal cancer

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Immunovirotherapy is an emerging cancer treatment approach that uses oncolytic viruses (OVs) as therapeutic agents. OVs infect and selectively replicate within cancer cells, inducing an immunogenic cell death and activating the immune system against the tumor. Despite OVs therapy having revolutionised the cancer treatment, inconsistent and short-term antitumor T-cell responses persist. Arming OVs with immunostimulatory cytokines or specific tumor-antigens represents a promising strategy to increase their efficacy and reduce clinical variability.

In this study, we generated and characterised an oncolytic adenoviral vector armed with interleukin-12 (Ad5/3D24_IL-12), a potent cytokine able to trigger a strong antitumor T-cell response. We evaluated the anticancer effect of Ad5/3D24_IL-12 decorated with specific tumor peptides, using the previously described PeptiCRAd vaccine platform.

We developed two Ad5/3D24_IL-12 decorated with either SIINFEKL or SYLPPGTSL antigens, respectively expressed in B16.OVA melanoma or CT-26 colorectal carcinoma cells. We evaluated vectors decorated with these antigens (PeptiCRAd_IL-12 platform) both *in vitro* and in syngeneic murine models of melanoma (C57BL/6J mice inoculated with B16.OVA cells) or colorectal carcinoma (Balb/c mice inoculated with CT-26 cells). Subsequently, we stimulated splenocytes obtained from treated mice with either SIINFEKL or SYLPPGTSL peptides, in order to assess the cytotoxic T-cell responses by IFN-γ ELISpot assay. Furthermore, we explored the phenotype of lymphocyte populations tumor-infiltrating and the tumour-draining lymph nodes collected from treated mice.

In melanoma and colorectal syngeneic murine models, treatment with PeptiCRAd_IL-12 significantly reduced tumor growth compared to PeptiCRAd-receiving mice, used as control group. After stimulation with the specific tumor antigen, we observed an increased and specific IFN-γ production by T-cells in splenocytes collected from PeptiCRAd_IL-12-treated mice, compared to the control group. Specifically, we observed an increased CD8+ T-cell infiltration and a reduction of the exhausted T-cell phenotype in the tumor microenvironment.

Based on our results, we have significant evidence adding IL-12 expression to the PeptiCRAd system increases a specific anticancer T-cell response in melanoma and colorectal cancer models.

P0998

Lentivector onco-targeting for solid tumor gene therapy

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Among the innovative therapeutic options in cancers refractory to current therapies such as pancreatic adenocarcinoma and metastatic prostate cancers, gene-based therapies show considerable promise. First, cancer gene therapy needs efficient gene transfer into the tumors. Lentiviral vectors pseudotyped with the broad tropism VSV-G envelope glycoprotein provide high transduction efficacy, as proven by the recently approved genetic disease and cancer *ex vivo* applications. Second, cancer gene therapy needs oncospecific transfer of the therapeutic genes, especially when bringing potentially harmful signals for healthy cells.

To obtain tumor-restricted oncotropism, we compared several pseudotyping solutions targeting cell surface antigens of pancreatic and prostate cancer models. First, we produced oncotropic lentiviral vector by engineering the E2 recognition glycoprotein of Sindbis virus (SINV-G), leaving intact the fusion E1 monomer. Second, we prepared lentivectors exposing both engineered SINV-G and low quantities of unmodified VSV-G. Third, we engineered the recognition part of the VSV-G, challenging the fusion activity. All the pseudotyping solutions used either biotinylated

monoclonal antibodies, biotinylated aptamers or the production of fusion proteins with their single-chain variable fragment (scFv).

Rhizavidin-SINV-E2 coupled with specific biotinylated antibodies was highly robust and flexible, systematically reaching efficiency, specificity and comparable performance with different targeted surface antigens. Biotinylated aptamers, although reaching acceptable transduction efficiencies did not outperform the antibody-based transduction. The scFv-SINV-E2 showed as efficient as the broad tropism VSV-G, but with exclusive specificity to cells expressing the targeted antigen. Moreover, with these envelope glycoproteins, the transduction efficiency was proportional to antigen expression by cancer cells, a crucial point when healthy cells display low expression of the targeted antigen. The high fusion capacity of the VSV-G increased scFv-SINV-E2/E1 transduction efficiency but abrogated specificity. Intra-tumor injections of lentiviruses displaying scFv-SINV-E2 in mice bearing subcutaneous tumors produced transduction rates as efficient as lentiviruses displaying VSV-G. Importantly, while VSV-G lentivectors injected intravenously delivered the reporter gene at the injection site, in the liver, and in the bone marrow, scFv-SINV-E2 lentivectors reached only the tumors and its metastasis and no other detectable site, confirming the strong therapeutic anti-cancer value of lentivector onco-pseudotyping. The VSV-G engineered glycoproteins are being evaluated for efficacy and specificity and 3D predicted models of the engineered glycoproteins are being built.

In conclusion, we confirmed oncotropic vectors engineered with antibodies or their variable fragments target only the cancer cells, with high reproducibility and applicability for *in vivo* transfer of therapeutic genes. Several modalities for viral glycoprotein engineering highlight the adaptability of lentiviral targeting to any tumor cell surface landscape. Considering the difficulty of reaching every tumor cell with toxic genes, this onco-specific targeting will be key for implementing intratumoral vulnerabilities.

P0999

Harnessing CRISPR/Cas9 to target cancer-specific genetic amplifications and activate anti-tumor immune responses

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Amplified oncogenes are prevalent in numerous cancers, acting as key drivers of tumorigenesis or tumor progression. Unique to cancer cells, these amplified oncogenes can be targeted through induction of DNA breaks, which could trigger programmed cell death, making them ideal therapeutic targets. However, the specific targeting of these amplified genes/regions remains a challenge. To address this, we introduce a novel CRISPR/Cas9-based strategy specifically designed to target oncogene amplifications with high precision and efficiency. This approach targets the amplified regions within cancer cells to ensure focused action.

Using in vitro and in vivo assays in a MYCN-amplified neuroblastoma model, we have demonstrated the efficacy and safety of our method. Our data shows a significant reduction in cell proliferation and improved survival rates in mice models. Moreover, our strategy robustly

activates the immune system – a critical component that significantly enhances the potential effectiveness of the therapy. By engaging the patient's immune cells, our approach directly targets the cancer cells and also bolsters the patient's immune mechanism to combat the tumor. This highlights the crucial role of immune system activation in our treatment and opens new avenues for developing selective and potent cancer therapies.

P1000

CRISPR/Cas13-mediated targeting of *MYCN* amplifications for selective cancer cells elimination in neuroblastoma

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Oncogene amplification is a common anomaly in cancer cells, playing a key role in the development and progression of many cancers, including neuroblastoma, a severe paediatric cancer. This process results in increased copies of specific tumour-associated genes, such as *MYCN*, which is associated with high-risk neuroblastoma cases. Despite recent advancements in cancer treatment, children with high-risk neuroblastoma have a five-year survival rate of only about 50%, facing a high risk of relapse and resistance to existing therapies.

In approximately 25% of neuroblastoma cases, *MYCN* amplification is detected and associated with an unfavourable prognosis. Studies have shown that reducing *MYCN* expression can promote apoptosis (programmed cell death), underscoring the potential of targeting MYCN protein as a therapeutic strategy. However, developing effective inhibitors for MYC proteins has proven challenging, highlighting the urgent need for innovative therapies with improved efficacy and fewer side effects.

Our recent research focuses on a novel approach using CRISPR/Cas13 technology to specifically target and destroy *MYCN*-amplified transcripts in cancer cells. We have demonstrated specific and efficient elimination of these cancer cells in vitro, along with significant reductions in tumour growth and mortality in animal models. Additionally, we have investigated the role of the Cas13 collateral effect in cancer cell death and the underlying molecular pathways. This selective elimination of neuroblastoma cells represents a potential breakthrough in developing advanced therapeutic options for this challenging cancer type.

P1001

Using primary cell culture models to study adenoviral vectors for virotherapy of head and neck squamous cell carcinoma

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Adenoviruses (AdVs) are investigated as oncolytic adenoviral vectors (OAdV) and for tumor gene therapy (TGT) and antitumor effects can be enhanced by expression of effectors such as gene editing tools or immune modulators. Head and neck squamous cell carcinomas (HNSCC) have a poor prognosis, are difficult to treat or resect and common treatments cause severe side effects. Moreover, a portion of HNSCC is HPV positive. As HPV oncogenes can complement OAdV replication, HPV related cancers potentially support and enable OAdV-mediated tumor lysis. Therefore, HNSCC are interesting targets for virus OAdVs therapy. As HNSCC are heterogeneous with regard to HPV status and other biomarkers and response to treatment, we aimed to address these individual differences between patients. Following tumor resection we established a pipeline for primary patient derived tumor cell cultures to study their response to AdVs. AdV vectors are mostly based on AdV serotype 5, having limited applicability due to high seroprevalence and strong liver tropism. Alternative AdV serotypes might have beneficial features and can be used as OAdV too. Using a library of reporter gene expressing AdV serotypes representing the natural diversity of human AdVs, we investigate the potential of alternative AdVs for oncotherapy of HNSCC. Transduction of individual primary cell cultures from patient tumors allowed us to identify suitable AdVs that can be used as OAdV or TGT vectors by comparing reporter gene expression and the effects of the different AdV serotype on tumor cell viability. HPV status and AdV receptor expression was investigated on primary tumors and on cells cultured from them, and disease-relevant patient information collected in order to correlate this with the outcome of AdV transduction experiments. This allows personalized prognoses for potential therapy of individual HNSCC with specific AdV serotypes. We observed differences from patient to patient and differences in transduction and cell killing efficiency between different serotypes. Nevertheless, AdV serotypes 35 and 37 showed superior transduction and gene expression in a majority of samples. Surprisingly AdV37 showed less antitumoral effects than AdV35 and could therefore be used as TGT vector whereas AdV35 might be useful as OdV. Therefore, AdV35 and AdV37 are currently being converted into OAdV/TGT vectors that will be further tested for virotherapy of HNSCC and other cancer entities. Interestingly AdV4 showed bad transduction and gene expression in the beginning but outperformed other serotypes at later time points in a part of the tumor cells indicating slow but strong virus replication. Note that this observation may also be exploitable for vector applications. Moreover, we currently examine the combination of OAdV treatment with radiation and chemotherapy to generate insights into potential synergistic, additive or adverse effects of such therapeutic combinations. With this, we hope to be able to evaluate the influence of pretreatments on the potential response towards viral tumor therapy.

P1003

Eradication of large established tumors in mice by a new oncolytic vaccinia virus inducing immunogenic tumor cell death

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Vaccinia viruses (VACV) are versatile therapeutic agents and different features of various VACV strains allow for a broad range of therapeutic applications. Modified Vaccinia virus Ankara (MVA) is a particularly altered VACV strain that is highly immunogenic, incapable of replicating in mammalian hosts, and broadly used as a safe vector for vaccination. Alternatively, Western Reserve (WR) or Copenhagen (Cop) are VACV strains that efficiently replicate in cancer cells and therefore are used to develop oncolytic vectors. However, the immune evasion capacity of WR or Cop hinders their ability to elicit antitumor immune responses, which is crucial for efficacy in the clinic. Here, we describe a new VACV strain named Immune-Oncolytic Vaccinia virus Ankara (IOVA), which combines efficient replication in cancer cells with induction of immunogenic tumor cell death (ICD). IOVA was engineered from an MVA ancestor and shows superior cytotoxicity in tumor cells. In addition, the IOVA genome incorporates mutations that lead to massive fusogenesis of tumor cells, which contributes to improved antitumor effects. In syngeneic mouse tumor models, induction of ICD results in robust antitumor immunity directed against tumor neo-epitopes and eradication of large established tumors. These data demonstrate that IOVA may serve as improved oncolytic vector.

P1004

Harnessing the Power of Multiomics from Fresh and Fixed Patient Sample Characterization

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The omics era has greatly expanded the repertoire of approaches available for researchers and clinicians to unravel the complexity underpinning human health: Next Generation Sequencing (NGS) approaches can characterize genomes, epigenomes, transcriptomes and proteomes. The analyses are critical to assess in individuals both pre- and post-treatment during therapeutic development and early-stage clinical trials. Peripheral blood mononuclear cells (PBMCs) offer a non-invasive approach that, when combined with omics tools, can provide a near holistic view of immune processes across patient cohorts. Meanwhile, Formalin Fixed Paraffin Embedded (FFPE) tissues are a staple in clinical diagnostics and an ideal means to store archival tissue but can be difficult to work with in traditional NGS assays.

Here we detail workflows using both fresh and fixed patient samples to rapidly produce a diverse set of multiomics results including genomics, epigenomics, transcriptomics and proteomics. For fresh blood draws, this starts with automated sample handling and processing to ensure high

viability and yield of PBMCs, along with simultaneous plasma separation and collection. Samples are then aliquoted and simultaneously processed for whole exome sequencing, single cell RNA sequencing, epigenetic characterization and Olink biomarker analysis. For fixed tissues, FFPE blocks were serially sliced into various FFPE slides, with a single slide H&E stained. Individual slides were then utilized for genome, epigenome, single cell RNA-seq, and digital spatial profiling. Genome information was captured using hybrid capture based approaches followed by deep NGS on an Illumina platform and analyzed for a variety of variants and tumor mutational burden. DNA methylation was detailed using target capture probes targeting DNA methylation sites. Single cell approaches were applied to explore the transcriptome using 10X Genomics scRNAseq kit, and Digital Spatial Profiling (DSP) was done using the NanoString GeoMx® Whole Transcriptome Atlas and immunostaining.

With these robust workflows, all these datatypes can be produced within days of fresh or fixed sample receipt using minimal sample amounts. High throughput integrative omics workflows, as described here, drive greater insights in human health, allowing for a rapid combined approach to address the biological questions at hand.

P1005

New intergenic sites within vaccinia virus genome for transgene insertion

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Vaccinia virus (VACV) holds great potential both as a vaccinating and oncolytic vector due to its capacity to stably express and incorporate exogenous DNA. However, the VACV genome is densely packed with coding and regulatory regions, and very few *loci* are described for transgene insertion without deleting functional VACV sequences. To solve this, the intergenic insertion site between *I8R* and *G1L* VACV genes was described and is successfully used for such a purpose. However, additional sites are needed for the construction of VACV vectors that package several therapeutic transgenes. In this work, we analyzed the VACV genome and identified three different intergenic *loci* with the potential to harbor transgenes without affecting the expression of endogenous VACV genes. To demonstrate the suitability of these *loci*, we constructed oncolytic VACV harboring an expression *cassette* for GFP in these intergenic sites and assessed transgene expression and the oncolytic properties of the recombinant viruses. We demonstrated that two of these sites are suitable for transgene insertion, since the replicative and cytotoxicity of the virus *in vitro* are not affected. Moreover, the inserted transgene is expressed at high levels, and the expression of up- and downstream genes is not diminished. In addition, the VACV genome demonstrated stable and the recombinant viruses showed similar pathogenicity compared to control viruses in mouse models. Our findings reveal new intergenic sites suitable for transgene insertion that may allow for the construction of candidate oncolytic VACV combining the expression of different therapeutic transgenes.

P1006

Mechanism exploration of miR-379 in breast cancer: from molecular insights to clinical application prospects

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Breast cancer remains a significant global health challenge, necessitating innovative therapeutic approaches to improve patient outcomes. MicroRNAs (miRNAs), including miR-379, play crucial roles in gene regulation. miR-379 is part of the chromosome 14 miRNA cluster (C14MC). Initial findings by our group discovered that miR-379 was significantly decreased in breast cancer tissues, with even lower expression observed in metastatic sites. Previous *in vitro* and *in vivo* studies demonstrated that enrichment of miR-379 inhibits cell proliferation and tumor growth by downregulating critical proteins such as COX2 and Cyclin B, suggesting a role as a tumor suppressor and highlighting therapeutic potential in breast cancer.

To explore the underlying mechanisms of the tumour suppressive effects of miR-379, we conducted a comprehensive proteomic analysis in a triple-negative breast cancer (TNBC) model. 4T1 murine breast cancer cells expressing luciferase (4T1-Luc) were transduced with miR-379 (4T1-379) and orthotopically injected into immunocompetent Balb/c mice. Tumor proteins were then extracted, digested, and analyzed using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The MS data were processed with MaxQuant and Perseus platform. The Perseus-processed LFQ data was then transferred to R programmer for further analysis. This proteomic analysis identified 600 differentially expressed proteins (DEPs), with 11 downregulated and 43 upregulated proteins showing significant changes ($|LogFC| > 1$, $p < 0.05$).

The DEPs were then functionally annotated for Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways. Functional enrichment results revealed that miR-379 regulates critical cellular processes, including angiogenesis, cell growth, adhesion, apoptosis, and metabolism. Pathway analysis further highlighted the involvement of miR-379 in key signaling pathways such as TGF-β and PI3K/AKT axes, which are pivotal in cancer progression and metastasis.

To further identify the targets directly regulated by miR-379 in this proteome, we performed a prediction targets analysis using bioinformatics tools such as TargetScan, miRDB, and miRTarBase. The intersection analysis of predicted targets and the DEPs identified the oncogene HSPA5 as a direct target of miR-379. Targeting HSPA5 has been shown to reduce expression of CD44, a well-known cancer stemness marker. Our functional enrichment analysis showed that HSPA5 may regulate the TGF-β signaling pathway, suggesting that miR-379 impacts this pathway and may inhibit cancer stemness by reducing CD44 expression.

In support of this, analysis of CD44 expression by immunohistochemistry (IHC) in the 4T1-luc and 4T1-379 tumours revealed significant loss of CD44 in the presence of elevated miR-379. These findings propose a novel miR-379-HSPA5-TGF-β1-CD44 axis as a mechanism for breast cancer suppression and drug resistance reversal, indicating the potential for miR-379 as a therapeutic agent.

In conclusion, proteomic mapping of miR-379-enriched tumors reveals a significant role in regulating various processes and signalling pathways associated with cancer progression. Our

research underscores the importance of further translational studies to fully elucidate the clinical utility of miR-379. The promising results from our proteomic and functional analyses highlight miR-379 as a potential innovative therapeutic target.

P1007

Novel Strategies to Combine Personalized Medicine and Immunotherapy to Target Resistance in Cancer

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Treatment of metastatic cancer requires the use of chemotherapy with multiple drugs, as resistance to single agents occurs almost universally. Combination strategies can associate two or more chemotherapy drugs with other modalities such as targeted therapies and immunotherapy. Innovative platforms to generate genetic models of tumor cells to test efficacy and safety of drug-target combinations are instrumental to find ways to overcome individualized resistance. Here, we review the most common approaches to target both tumor cells and microenvironment by using cell and gene therapies. We describe various examples to combating multidrug-resistance in head and neck cancer and lung cancer, including studying the druggability of targets with AI-based tools, developing genetic cell models by genome editing, and high-throughput sinsibility testing of agents that engage the immune system metabolism to overcome mechanisms of resistance.

P1008

Non-viral HDR approach to correct two frameshift mutations in the COL7A1 gene

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Recessive dystrophic epidermolysis bullosa (RDEB) is a skin fragility disease caused by loss of functional type VII collagen (C7), a dermo-epidermal adhesive protein, leading to chronic blistering, progressive fibrosis and cancer. A premature termination codon-causing mutation in exon 80 of *COL7A1* gene, c.6527insC, is highly prevalent (close to 50% of pathogenic alleles) in the Spanish RDEB patient population. Similarly, two nucleotides deletion of in Exon 73 (c.6041_6042delAG) generates a frameshift mutation that also turns into null C7 expression. We have developed non-viral gene editing strategies based on homology directed repair (HDR) in both loci. The strategy is based on CRISPR/Cas9 electroporation, to generate double strand brakes, and small double stranded DNA molecules as template (300bp). Preliminary data in immortalized patient-derived keratinocytes showed efficacies above 80% near the gRNA cleavage

point. However, HDR efficacy decayed with distance making the system a good alternative for rewriting small regions (30bp around the cleavage). Taking advantage of this narrow window of action, we design gRNA that specifically recognize the patient mutation (not recognizing either the template or the wild-type sequence). Moreover, this strategy have demonstrated being feasible in primary patient derived keratinocytes for Exon 73 mutations with efficacies above 70%. In conclusion, this new set up could be a good alternative for HDR avoiding the use of viral vectors.

P1009

Benefits of bronchial epithelial repair by iPSCs in COPD

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COPD is a disease influenced by the environment and the epigenetic modifications it causes. This pathology could be the result of a profound dysfunction of epithelial repair. We aim to improve the repair of COPD airway epithelium by grafting pre-differentiated induced pluripotent stem cells (iPSCs) from the same patient. Epithelial cells were obtained from bronchial biopsies and were cultured in air-liquid interface (ALI). Pseudostratified native bronchial epithelia were mechanically damaged under different conditions. iPSC-derived epithelial cells were generated from the blood of the same patients and modified to express or not express GFP. Cells from reprogrammed IPS were differentiated to the early maturation stage vAFE (ventral Anterior Foregut Endoderm) and were added to the lesion level of native damaged epithelia. GFP expression was observed by fluorescence optical microscopy from the day of transplantation and for 42 days to follow repair of the lesion and to assess differentiation of transplanted cells. The culture wells were then fixed with formaldehyde to carry out immunofluorescence markings (GFP, eCAD, p63). GFP cells derived from vAFE-stage iPSC were successfully transplanted to the site of the ALI epithelial lesion. This was confirmed by observation of GFP under a fluorescence microscope. The detection of fluorescence on day 3, which intensified until day 7, was still present on day 42, underlining the long-lasting viability and stability of the transplanted cultures. Immunofluorescence staining at day 7 also demonstrated that GFP+ cells formed intercellular junctions (eCAD) with GFP- cells. Some GFP+ cells were also found to be p63+. It is possible to repair the pathological epithelium of a COPD patient using iPSC-derived epithelium. The repair mechanisms could provide clues to therapeutic avenues, targeting epigenetics and cell therapy?

P1010

A novel AAV gene therapy strategy targeting the liver to treat bone and dental defects in X-linked hypophosphatemia

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X-linked hypophosphatemia (XLH) is the most common form of genetic rickets (1/20 000 births) and is associated with elevated levels of fibroblast growth factor 23 (FGF23) leading to phosphate wasting and reduced active Vitamin D (1,25-$(OH)_2$-vitamin D) synthesis in kidneys. Mainly diagnosed during childhood by growth retardation and deformities of the lower limbs, this disorder also affects dentoalveolar mineralization resulting in higher susceptibility to spontaneous dental necrosis and periodontitis. Adult patients present also enthesopathy and muscular weakness affecting their quality of life. A FGF23-neutralizing antibody treatment, burosumab, was approved for clinical use as an alternative to the conventional treatment based on lifelong daily intake of phosphorus and 1,25-$(OH)_2$-vitamin D. Based on the central role of overactive FGF23 pathway in the pathophysiology of FGF23, we recently developed a liver-targeted AAV gene therapy strategy to bypass bone targeting and inhibit FGF23 pathway to rescue bone pathology with a single intravenous injection. The approach is based on the competition of the C-terminal FGF23 (cFGF23), secreted by hepatocytes in the bloodstream, on the binding of FGF23 to its renal receptor. This strategy, evaluated in Hyp-Duk mice, a murine model of XLH, demonstrated efficacy in the correction of phosphate wasting in kidney as well as correction of skeletal manifestations.

Here, we further refined the evaluation of the efficacy of the AAV treatment by evaluating its impact on the development of enthesopathies and the dental phenotype. AAV-cFGF23 was injected in 4-week-old Hyp-Duk mice and PBS-injected Hyp-Duk mice and WT littermates were used as controls (n=4-6 per group). Gene therapy efficacy was analyzed by micro-computed tomography (micro-CT) and histology. Three months after injection, AAV-cFGF23 treatment significantly reduced osteomalacia, restored bone microarchitecture parameters and enhanced long bone growth. Bone and joint alterations observed in Hyp-Duk mice were also improved, with a complete correction of sacroiliac joint alteration and a rescue of alkaline phosphatase activity level in Achilles tendon entheses in AAV-cFGF23-injected Hyp-Duk mice. In addition, dentoalveolar features of the disease were significantly corrected after AAV-cFGF23 injection. The restoration of alveolar bone volume fraction was associated with increased mineral density compared to untreated Hyp-Duk mice. A robust dental tissue response to AAV-cFGF23 was observed with an improvement in dentin/cementum volume comparable to WT levels and a decrease in first molar pulp volume compared to PBS-injected Hyp-Duk mice. Histological analyses highlighted the positive outcomes of the treatment on mineralization of different dental tissues such as dentin, alveolar bone, and also on tooth anchorage showing a restoration of periodontal ligament attachment in treated Hyp-Duk mice. Our data support the efficacy of the liver-targeting gene therapy approach to treat skeletal and dental manifestations of XLH at the systemic level. Given that XLH can be considered a prototypical bone disease, the success

achieved with our gene therapy approach in XLH opens the way to the extension of similar approaches in other skeletal disorders.

P1011

AAV-mediated KLF4 in lung endothelial cells as a treatment for pulmonary hypertension

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Pulmonary hypertension (PH) involves an increased resting mean artery pressure, resulting in right ventricular failure. On the cellular level, PH is characterized by endothelial dysfunction, endothelial-to-mesenchymal transition, smooth muscle cell proliferation, and exacerbated pro-inflammatory response. Krüppel like factor 4 (KLF4) is a transcription factor that maintains endothelial cell function and limits inflammation. We aimed to overexpress KLF4 in lung endothelial cells and evaluate the effect in a mouse model of pulmonary hypertension driven by hypoxia. ECs were treated with targeted AAVs overexpressing either KLF4 or EGFP as control. Markers of endothelial-to-mesenchymal transition were assessed by qPCR. Mitochondrial function was determined by live cell imaging, while tight junction integrity was analyzed by immunocytochemistry and ECIS(R). KLF4 overexpression in vivo was achieved in a murine hypoxia model of PH using a previously described pulmonary endothelium-targeted AAV2-ESGHFY variant. Two weeks after treatment, mice were subjected to hypoxia for a further 3 weeks. Right ventricular function was analyzed by echocardiography, while right ventricular systolic pressure (RVSP) was determined by right heart catheterization. In vitro, KLF4 overexpression halted hypoxia-induced endothelial-to-mesenchymal transition and limited inflammation. Moreover, tight junction protein ZO-1 was significantly increased following treatment with AAV-KLF4 as compared to controls. Moreover, mitochondrial function was rescued by KLF4 overexpression under hypoxic conditions. Importantly, targeted KLF4 overexpression in lung arterioles led to ameliorated cardiac function and decreased RVSP as compared to AAV-EGFP treated animals.

Taken together, KLF4 overexpression leads to improved right ventricular function and decreased inflammation due to its beneficial effect on tight junction integrity and the preservation of mitochondrial function in endothelial cells under hypoxic conditions.

P1012

Combined cellular and gene therapy to treat primary ciliary dyskinesia

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Primary Ciliary Dyskinesia (PCD) is a genetic disease caused by mutations that alter cilia beating, including in the respiratory airways, resulting in impaired mucus clearance and severe morbidity as well as increased mortality. We hypothesized that we could restore bronchial ciliary beating with genetically corrected iPSC differentiated into air-liquid interface bronchial epithelium model (iALI) for autologous cell therapy. The differentiation of iPSC in iALI is well established within the team. Moreover, we already shown that corrected cells generated from a PCD patient IPS cell line can be differentiated in iALI with functional ciliary beating. The aim of the project now is to assess the engraftment ability of the corrected cells and the repair of the pathological model after engraftment. Different issues have to be considered: the characterization of competent cells for bronchial engraftment, the study of different strategy for previous erosion of the bronchial epithelium and the assessment of the ciliary beating recovery to assure bronchial repair. We use a GFP-iPSC cell line generated in the lab to engraft on the corresponding control cell line and mutated cell line differentiated in iALI to answer these issues. Our results suggest that lung progenitors at the ventralized anterior foregut endoderm stage could be the most efficient cells for engraftment. Their self-renewal ability and their capacity to differentiate in the different cell type spectrum of the bronchial epithelium are promising for the development of a long term and efficient therapy. Concerning the bronchial erosion, we considered it necessary to promote cell engraftment because of the barrier function of the intact bronchial epithelium and the lack of selection advantage from the corrected cells. Different strategies, chemical or enzymatic, seem to provide good results but we need to assess the safety of each of them for *in-vivo* application. Finally, several experimental conditions allowed to observe GFP engrafted cells expressing cilia, suggesting that the grafted progenitors differentiated in ciliated cells. Functional recovery still needs to be confirmed. Next step of the project is to develop the therapy for *in-vivo* application, assessing the safety and efficiency of the graft in immunodeficient mini-pig model.

P1013

Papillomavirus As A Novel Viral Vector For Gene Therapy Of Olmsted Syndrome

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Olmsted syndrome is a rare dominant genetic skin disease caused by a gain of function point mutation in the Transient receptor potential vanilloid-3 (Trpv3) gene. TRPV3 is a non-selective cation channel sensitive to non-noxious warm temperatures (31-39°C) mainly expressed in keratinocytes and sensory neurons. The mutation renders the channel hyperactive, thus increasing intracellular calcium levels; this leads to hyperproliferation and defects in the

maturation of basal keratinocytes, invasion of immune cells, chronic itch and pain. Olmsted syndrome, given its well-defined etiology, stands out as a promising target for gene therapy.

We propose Papillomavirus as a new viral vector for skin gene therapy: papillomavirus has a natural tropism for continuously dividing basal keratinocytes, as it can infect only actively dividing cells, this is because the virus can enter the nucleus only during mitosis. Papillomavirus is a non-enveloped virus that can package up to 8 kilobases of double-stranded DNA. The delivered genetic material typically remains episomal. These characteristics make papillomavirus a promising candidate for delivering gene therapies based on genome editing. It can deliver much larger constructs, such as base editors or large Cas9 proteins, which cannot fit into Adeno-Associated Viruses (AAVs) that can package only up to 4.7 kilobases. Papillomavirus also has an advantage over lentiviruses, as it does not integrate into the host genome. For genome editing, non-integration is preferable to avoid continuous production, unintended off-target effects, and the risk of disrupting host genes.

To demonstrate the efficacy of PapillomaVirus Like Particles (PVLPs) as a viral vector for gene therapy we employed PVLPs to deliver SaCas9 with a gRNA to disrupt Trpv3 locus. We transduced both a keratinocyte cell line (KERA-308) and mouse primary keratinocytes with PVLP-SaCas9, achieving indel formation in more than 50% of the alleles ten days post-transduction. We then assessed the functionality of the disrupted channel using FURA2-AM calcium imaging. Ten days after transducing keratinocytes with PVLP-SaCas9, there was a significant reduction in response to carvacrol, a TRPV3 agonist. A disrupted Trpv3 should be preferable over the mutated gene since genetic ablation of Trpv3 in mice leads to only mild phenotypes such as a thinner stratum corneum and an altered wavy fur.

Furthermore, we created skin equivalents from PVLP-treated primary keratinocytes to demonstrate the applicability of this method to autologous transgenic skin transplants. Editing the genome with Cas9 is a one-time event passed down to cell progeny; we modeled this using Cre recombinase on primary keratinocytes from a (Lox-STOP-Lox) LSL-TdTomato transgenic mouse. Transducing primary keratinocytes from an LSL-TdTomato mouse with PVLP-Cre, we successfully generated skin equivalents that were almost completely recombined.

Thus, we propose PapillomaVirus Like Particle as a promising alternative vector for ex vivo gene therapy of the skin.

P1014

Characterization of mesenchymal stromal cells subpopulation with enhanced survival capacity upon intradermal injection

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Patients with Recessive Dystrophic Epidermolysis Bullosa (RDEB) suffer from severe blistering and complications due to mutations in *COL7A1* encoding type VII collagen (C7). Mesenchymal Stromal Cells (MSC) show promise in improving wound healing and reducing skin inflammation in RDEB patients owing to their ability to express C7 as well as their anti-inflammatory properties. Our objective was to refine *in vitro* conditioning of human bone marrow-derived MSC (hBM-MSC) and evaluate their fate upon local injections in murine models. First, hBM-MSC from healthy

donors were transduced with a lentiviral vector encoding firefly luciferase and mCherry reporter proteins, without altering their MSC phenotype. Transduced hBM-MSC were then subjected to various culture conditions before being administered intradermally (ID) in immunodeficient mice. Their *in vivo* survival was assessed using the bioluminescent reporter through *in vivo* imaging. Previous results have shown 4 months of survival of ID-injected BM-MSCs cultured under standard *in vitro* culture conditions. Surprisingly, although most injected cells died within the first two months in all tested conditions, a small population (10%) of live bioluminescent cells persisted for at least one year post-injection. We analyzed the hBM-MSC populations cultured under the different conditions prior to injection through single-cell RNA sequencing. In parallel we sampled murine skins injected with the hBM-MSC two months post injection and analyzed the surviving subpopulation by immunostaining and spatial transcriptomic. Our results indicated that the surviving cells were initially present in the injected cell population and originate from the same cluster. ScRNAseq analyses demonstrated that culture conditions have an impact on the expression of genes involved in wound healing and immunomodulation. Moreover, spatial transcriptomics data indicated that these surviving cells maintained the expression of *THY1*, *ENG* and *NT5E in vivo* and shared several characteristics with cutaneous fibroblasts. They expressed *COL7A1* and showed enriched expression enrichment of genes involved in extracellular matrix structural constituent, collagen fibril organization, collagen binding, and growth factor binding which have substantial therapeutic value for wound healing. This comprehensive analysis allowed us to identify a distinct subpopulation of BM-MSC capable of long-term survival following local injection and to determine their phenotype. This holds promise for improving cell therapy protocols for clinical translation in RDEB persons.

P1015

Long-term *ex vivo* hematopoietic stem cell expansion as a tool to improve the feasibility of gene therapy in osteopetrosis

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Autosomal recessive osteopetrosis (ARO) is a rare genetic disease, that affects osteoclast function leading to death in the first decade of life. Most ARO patients present mutations in *TCIRG1* gene, necessary for bone resorption. Osteoclast dysfunction results in bone marrow (BM) failure, limited BM cavity and increased number of circulating CD34+ cells. The standard treatment is the hematopoietic stem cell (HSC) transplantation, but its success is limited by several constraints and the severity of the disease. Gene therapy (GT) represents another therapeutic option, because it could minimize the immune-related complications of allogeneic transplant. *Ex vivo* hematopoietic stem and progenitor cell (HSPC) expansion has been suggested to improve the hematopoietic reconstitution in low-cell dose grafts. Here, we coupled the HSC expansion protocol with GT. For the applicability in osteopetrosis, we set up the protocol starting from the spleen of wild type (wt) 15-day-old mice. Sorted HSPCs ($Lin^-Sca1^+c\text{-}Kit^+CD34^-CD150^+$) were cultured in cytokine-supplemented serum-free medium at two different O_2 concentrations (5% vs. 20% O_2). We observed up to $1x10^5$-fold cell expansion and monitored phenotype by FACS analysis. Cells cultured with high O_2 were characterized by a higher number of differentiated cells (up to 5% of LSK cells), while 5% O_2 culture cells maintained the expression of primitive LSK markers (50%).

Subsequentially, we transduced cells using reporter PGK.GFP lentiviral vector (LV) and the day after we performed the colony-forming unit assay to assess the HSPC output. We detected comparable GFP expression and vector copy number (VCN) in the liquid culture and in colonies, without any impact on cell phenotype. Interestingly, the VCN was higher in cells cultured in low O_2(7.4 in low O_2 and 4.2 in high O_2 conditions). After 20 days of expansion *in vitro*, we transplanted conditioned CD45-mismatched wt recipients with various doses of day0 HSC equivalents, to assess the long-term engraftment potential of HSC expanded cultures. We observed higher donor engraftment in mice transplanted with 5% O_2 expanded cells. Stable GFP+ expression was detected in tissues (peripheral blood, BM, spleen and thymus) regardless of the different culture conditions, showing that the transduction does not impact on the fitness to engraft long-term. Then, we tested the *ex vivo* HSC expansion in the *oc/oc* mice, the murine model of *TCIRG1*-osteopetrosis, using low O_2 condition. We transduced expanded HSPCs from the spleen of *oc/oc* mice with therapeutic PGK.TCIRG1 LV. We observed up to 1.7x10^5-fold cell expansion. *In vitro* analysis showed comparable results to those obtained from splenic wt HSPCs. After the *ex vivo* expansion, we transplanted conditioned *oc/oc* neonates and the *in vivo* experiment is ongoing. GT-treated *oc/oc* mice survived longer than their expected lifespan and showed amelioration of the disease phenotype. In conclusion, we observed that the low O_2 culture maintains more primitive HSC phenotype. We demonstrated that HSPC expanded cultures can be efficiently transduced *in vitro* without impacting their phenotype and engraftment potential. The long-term *ex vivo* HSPC expansion protocol may improve the feasibility of gene correction to treat those diseases, like Osteopetrosis, in which limited HSPC numbers are available.

P1016

Hematopoietic stem cell-based gene therapy in Mucopolysaccharidosis type I Hurler (OTL-203): focus on skeletal damage and cross-correction mechanisms

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Mucopolysaccharidosis type I Hurler (MPSIH), a rare genetic lysosomal storage disease, is caused by deficiency of the alpha-L-iduronidase (IDUA) enzyme resulting in glycosaminoglycans (GAGs) accumulation in multiple organs. In the skeleton, the impaired GAGs degradation leads to a progressive and severe skeletal dysplasia, also known as dysostosis multiplex, that is not fully addressed by the standard of care with hematopoietic stem cell transplantation (HSCT), resulting in high unmet clinical need. Supported by preclinical findings in the MPSI murine model, 8 MPSIH patients were treated in a phase I/II clinical trial (NCT03488394) with ex-vivo hematopoietic stem cell gene therapy (HSC-GT, also known as OTL-203) showing early beneficial effects on the skeletal phenotype, evaluated in terms of clinical, functional and radiological parameters up to 4

years post-treatment. However, as the molecular mechanisms of skeletal damage and cross-correction after HSC-GT are not completely elucidated, we investigated the skeletal compartment's key players, i.e. mesenchymal stromal cells (MSCs), chondrocytes (CHs), osteoblasts (OBs), and osteoclasts (OCs) of MPSIH patients enrolled in the trial before and after HSC-GT. Patient-derived MSCs and OCs characterization showed similar features as compared to healthy donor (HD)-counterparts. Although patient-derived MSCs normally underwent the in vitro intramembranous ossification, patient-derived OBs showed intracellular GAGs accumulation, with unknown effects on their functionality. OCs and their supernatants obtained from HSC-GT treated MPSI patients demonstrated supraphysiological enzyme activity. Exposing patient-derived OBs to gene-corrected OCs supernatant reduced the intracellular GAG accumulation, due to the ability of OBs to uptake the IDUA enzyme through the mannose-6-phosphate receptor, showing the feasibility of the cross-correction. An *in vitro* scaffold-free 3D model, used to generate hypertrophic CHs, revealed a reduced expression of Collagen type X and Sox9 in patient-derived CHs as compared to HDs. The *in vivo* subcutaneous implantation of the 3D chondrogenic pellets into immunodeficient MPSI mice models the later phases of the endochondral ossification process, which will allow us to study the skeletal remodeling process in an enzyme-deficient environment. Furthermore, a deep analysis of the skeletal disease and correction after HSC-GT was performed in the MPSI immunocompetent murine model treated with HSC-GT showing a rescue of relevant disease-associated alterations, such as osteocytes and periosteum vacuolation. Vacuolation of chondrocytes of the growth plate and the articular cartilage were improved, together with histomorphometric analyses and pQCT long bones trabecular and cortical parameters. The skeletal alterations found in MPSI mice growth plates, trabecular bone and Achilles tendons appeared early in life, as two months of age, and progressed over time, supporting the need of an early intervention. Finally, the histological analyses of the osteomedullary biopsies of MPSIH patients before HSC-GT revealed vacuolation of the enthesis tissue, which was completely rescued after HSC-GT. These findings obtained by in vitro, in vivo and ex vivo studies suggest a beneficial effect of HSC-GT on characteristic MPSIH skeletal disease-associated features.

P1017

Single cell transcriptome of spontaneously circulating hematopoietic stem/progenitor cells supports lentiviral mediated gene therapy for *TCIRG1*-osteopetrosis

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Under physiological conditions, hematopoietic stem and progenitor cells (HSPCs), positive for the CD34 marker, are known to recirculate at low levels in the peripheral blood for immune-surveillance and hematopoietic homeostasis. Patients affected by *TCIRG1*-defective autosomal recessive osteopetrosis (ARO) show a distinctively high frequency and counts of spontaneously circulating HSPCs, due to their defective bone marrow niche. This rare genetic disease is characterized by defective osteoclast resorptive functions, leading to dense bones, progressive reduction of BM space and finally to BM failure in infancy. The standard of care is allogeneic hematopoietic stem cell (HSC) transplantation, while lentiviral vector (LV) gene therapy (GT) may represent a valuable alternative. However, GT protocols must rely on the *ex vivo* manipulation of circulating CD34+ (cCD34+) cells, which are the only source available in ARO patients. We demonstrated that ARO cCD34+ can be corrected by a *TCIRG1*-expressing LV and expanded with a UM171-based protocol. However, differently from drug-mobilized HSPCs, ARO CD34+ cells are regularly exposed to normoxia and inflammatory molecules that can impact on their stemness and fitness. We performed single cell RNA sequencing on cCD34+ from 3 ARO patients, in comparison to healthy donor (HD) cord blood (CB)-derived CD34+ cells, to dissect HSPC heterogeneity at steady state and after *ex vivo* GT and expansion. Applying state-of-the-art pipelines, we identified 20 clusters, spanning from the most primitive HSCs to more committed erythroid, myeloid and lymphoid progenitors. At steady-state, ARO cCD34+ cells contain primitive HSCs and multipotent progenitors (MPPs) similarly to HD, but they showed lower content of megakaryocyte/erythroid-biased MPPs in favour of lympho-myeloid primed progenitors. In addition, we observed high frequency of B, dendritic and granulo-monocyte cell progenitors. These results suggest that in ARO, the lack of adequate BM niches induces circulation of populations normally retained within the BM. After UM171-mediated expansion, we observed maintenance of primitive HSC and MPP subsets in both ARO and HD CD34+ cells, in line with the long-term engrafting and repopulating capacity of the expanded product that we observed in NSG mice. UM171-expansion also resulted in an absolute increase of myeloid progenitors, that will contribute to the differentiation of gene-corrected osteoclasts in ARO patients after GT. Notably, no skewing of subset composition was observed after LV transduction.

In conclusion, single cell transcriptomic analysis of ARO cCD34+ reveals the peculiar features of HSPC in this pathological condition and endorses their use for LV GT. Detailed differential gene expression and pseudotime analyses will provide relevant data to optimize therapies for ARO patients but could also suggest novel pathways regulating CD34+ egress from hematopoietic niches.

P1018

Efficient *in vitro* correction of a highly recurrent *COL7A1* pathogenic variant using Cytosine Base editing to treat recessive dystrophic epidermolysis bullosa

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Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a rare and severe genetic skin disease resulting in blistering of the skin and mucosa after minor trauma. RDEB is caused by a wide

variety of variants in *COL7A1* encoding type VII collagen, the major component of anchoring fibrils that form key attachment structures for dermal-epidermal adhesion.

Here, we achieved highly efficient *COL7A1* correction in primary RDEB cells using Cytosine Base editors (CBEs), which targeted the non-edited strand and mediated the conversion of cytidine to uridine, resulting in a C-to-T substitution to correct the targeted nucleotide.

For this study, we designed four guide RNAs (gRNAs) targeting the c.425A>G (p.K142R) variant. This is a highly recurrent variant, located at the last nucleotide of exon 3, which leads to several altered splicing events resulting in premature stop codons.

Four *in vitro* transcribed CBEs together with four chemically modified gRNAs were delivered as mRNA by nucleofection in RDEB keratinocytes and fibroblasts (K and F). Among the gRNAs and CBEs tested, two gRNAs showed up to 73 and 91% editing in RDEB cells at the gDNA level, as evaluated by Sanger and high-throughput sequencing. RT-PCR and sequence analysis showed the presence of a correct transcript in gene-edited RDEB cells. Assessment of C7 protein expression and secretion after editing revealed levels of restored C7 similar to C7 in normal human cells. Rescued C7 protein expression was also confirmed by immunofluorescence staining of primary RDEB-K and F, in contrast to untreated RDEB cells which completely lacked C7.

Evaluation of off-target activity of the most active gRNA in RDEB cells showed no evidence for non-specific cleavage activity at *in silico* predicted sites. We concluded that *COL7A1* editing and desired phenotypic correction (up to 91%) could be achieved in primary RDEB-K and F by Base editing using combined gRNA- and CBE mRNA delivery.

Grafting of genetically corrected 3D skin equivalents onto nude mice is ongoing to demonstrate functional correction for future *ex vivo* clinical applications.

P1019

Development of an AAV9-based gene therapy for dermatosparaxis Ehlers-Danlos syndrome

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Ehlers-Danlos syndrome (dEDS) is an autosomal recessive collagen disorder caused by a loss of function mutation in ADAMTS2. The mutation leads to impaired extracellular cleavage of the N-terminal-propeptide of type I procollagen by the procollagen N-proteinase enzyme. This results in collagen fibrils with aberrant hieroglyphic morphology and decreased tensile strength. We chose this form of EDS to develop a gene therapy because 1) it is caused by a loss of function of a secreted protein (ADAMTS2), 2) it is encoded by a 3.6kb cDNA, and 3) it is a ubiquitously expressed protein with a immature procollagen target with no known function. We hypothesized that global secretion of ADAMTS2 will be efficacious in treating this form of EDS.

Due to the extracellular function of ADAMTS2, our strategy is liver and skeletal muscle directed AAV gene transfer with systemic secretion of ADAMTS2. Four AAV9 vectors were designed and packaged: 1) encodes for a wildtype signal peptide and codon optimized ADAMTS2 cDNA 2) encodes for an enhanced signal peptide (sp7Δ8) and a codon optimized ADAMTS2 with an HA tag at the c-terminus of the cDNA 3) a codon optimized ADAMTS2 cDNA with an HA tag located

at the C-terminus of the cDNA or 4) in the middle of the cDNA. Vectors were tested in wild type mice for expression and toxicity (n=4 WT 6-week-old C57BL/6 mice per group). Mice were treated via intravenous tail vein injection at a dose of 1E12 vg/mouse and were sacrificed 1-month later. No signs of toxicity were observed.

A knockout ADAMTS2 mouse model was used for further therapeutic development. Two cohorts of mice were injected by intravenous tail vein injection at a dose of 1E12 vg/mouse at 8-weeks of age with three vectors containing an HA tag in the middle of the cDNA and a wild type signal peptide (n=9), an HA tag at the C-terminus of the cDNA and an enhanced signal peptide for increased secretion from liver and skeletal muscle (n=8), and a wild type signal peptide and no HA tag (n=8). Wild type untreated controls (n=7) and untreated ADAMTS_KO mice (n=4) were also evaluated. Mice were sacrificed at either 8 months or 6 months post injection. Transmission electron microscopy of the skin in longitudinal and cross-section revealed no difference in the amount of abnormal hieroglyphic collagen between treated and untreated mice. Histopathological assessment of all major tissues is currently underway by a boarded veterinary pathologist. Vector genomes were performed on the liver, quadriceps femoris and kidney. Anti-HA Western blot revealed a unique band at approximately 135 kDa that we theorize represents the 132 kDa isoform of ADAMTS2 in addition to the 2x HA tag in the quadriceps femoris of mice treated with the enhanced signal peptide (sp7Δ8) vector with the HA tag on the C-terminus of the C-DNA but was not present in the other vector groups. Additionally, purification of type I collagen from mouse skin followed by SDS-page and Coomassie blue demonstrated a decrease in immature pna1 collagen in treated compared to untreated mice.

P1020

Optimization of gene insertion strategies for restoration of CFTR expression in airway epithelium

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Our objective is to develop a universal gene editing strategy capable of curing individuals with Cystic Fibrosis (CF). CF can be caused by a diverse set of mutations distributed across the 250 thousand base pairs of the *CFTR* gene. Because of the large number of mutations already reported, there is a strong interest in directly targeting either a partial or full-length *CFTR* cDNA into the endogenous *CFTR* locus, rather than developing mutation-specific approaches. This strategy has the advantage of correcting or compensating for all *CFTR* mutations downstream of the integration site. If this can be achieved while retaining the native *CFTR* chromatin structure and regulatory sequences, it also has the possibility of restoring appropriate cell-type specific expression. We have previously demonstrated the ability to efficiently target integration of AAV-delivered partial *CFTR cDNAs* into *CFTR* introns 7 or 8 of CF airway basal cells with restoration of CFTR expression and function in the derived airway epithelium. To correct for all or nearly all *CFTR* mutations, it likely would be necessary to target integration of the partial *CFTR* cDNA sequences into the most upstream regions of *CFTR* sequences. In this regard, we are seeking to develop a single donor vector capable of efficient *CFTR exons 2-27 (CFTR2-27)* integration into intron 1 (employing a synthetic splice acceptor [SA]) or exon/intron 2 (utilizing the native exon 2 SA) enabling appropriately regulated CFTR expression.

We have recently performed a comparative evaluation of targeted integration into a variety of sites distributed across intron 1, exon 2 and intron 2 with the goal of identifying target sites that can be efficiently targeted -- and transgene constructs that give rise to robust expression. We

incorporated two reporter cassettes in the donor construct, *pgk-mScarlet* for selection of targeted cells and a chimeric *CFTR-luciferase* to read out levels of expression. This enabled us to perform a comparative assessment of levels of transgene expression in fully differentiated airway epithelia derived exclusively from targeted cells. Our study has revealed clear differences in expression level as a function of integration site -- further modulated by the choice of poly adenylation (polyA) sequences. For those integration sites and polyA sequences exhibiting highest luciferase expression, we have also been evaluating delivery and expression of the desired *CFTR2-27* transgene. Since some of the transgene constructs exceed AAV packaging limits, we have been evaluating alternative strategies to deliver *CFTR2-27*.

Although this project is being performed in the context of AAV-mediated delivery and homologous recombination-mediated integration, we anticipate that this examination will be highly informative irrespective of the delivery vector used or the precise method of integration. Furthermore, we are interested in evaluating how these findings in ex vivo can be translated into direct in vivo targeting of CF airway epithelium.

P1021

Skin regeneration by physical stimulation based on identification of dermal stem-cell reservoir

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Skin, the most outermost layer of the body, physically protects internal organs from environmental insults and helps maintain the body shape. The physical properties of skin depend upon the dermal layer, which consists of abundant collagen fibers produced and regulated by fibroblasts. Thus, deterioration of the condition of fibroblasts with aging or under UV irradiation damages collagen fibers and impairs the physical properties of the skin, which leads to skin ulcers and delayed wound healing, as well as wrinkles and sagging. However, there are few impactful approaches to improve this situation. One possibility would be activation of stem cells in the dermal layer, which are reported to exist around blood vessels or scattered in the dermal layer, but they also decrease with aging, so that it is difficult to utilize them.

Therefore, in this study we firstly tried to identify the presence and distribution of stem cells in aged skin by immunohistochemical evaluation of stem cell markers in human surplus skin from plastic surgery, obtained with permission. We found that stem-cell-like cells positive for nestin, CD34, and CD54 exist in the dermal layer around sebaceous glands. Although the size of sebaceous glands decreases with aging, they are still present in aged skin, and stem cell marker-positive cells are still present. Further, although collagen fibers are degraded in the dermal layer of aged skin, the fibers still remain around sebaceous glands. Fibroblasts in the young dermal layer, observed by 3D electron microscopy (serial block-face scanning electron microscopy: SBF-SEM) showed well-spread dendrites, indicating that are active and producing collagen fibers, whereas fibroblasts in the aged dermal layer become round and inactive, with decreased collagen production. However, fibroblasts around sebaceous glands remained spindle-shaped even in aged skin. These results suggest that sebaceous glands maintain stem-like cells in the dermal layer even in aged skin, and we hypothesized that this could be the key to regenerating the condition of the dermal layer of aged skin.

We then investigated methods to stimulated stem cell marker-positive cells around sebaceous glands. We screened a variety of physical stimulation methods, using organ-cultured skin, and found that pressure on to the skin from the surface side (30% deformation for 7days) increased the stem cell marker-positive cells around sebaceous glands. Spindle-shaped active fibroblasts were also increased around sebaceous glands, as was the amount of collagen fibers.

Therefore, sebaceous glands could act as a stem cell reservoir in the dermal layer. Further, physical stimulation of the skin appears to be a useful approach to induce proliferation of stem cell marker-positive cells around sebaceous glands in order to increase active fibroblasts, increase collagen production, and improve the impaired physical properties. These changes are expected to contribute to better clinical outcomes and quality of life.

P1022

Redefining cell therapy: developing a cost-effective microencapsulation technique for global impact

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Cell therapy has shown promising results in treating various diseases and injuries by aiding tissue repair and regeneration. However, this approach faces significant challenges, particularly in the effective introduction of new cells into patients due to immunological clearance and limited cell survival. Overcoming these obstacles is crucial to maximize the therapeutic potential of cell therapy. Encapsulation techniques using biocompatible materials have been employed to provide mechanical protection and a stable environment for the cells. Additionally, microfluidic systems enhance this approach by offering precise control over capsule shape and size, making cell encapsulation more efficient and reliable. We present an innovative, simple, and cost-effective system called Air-Jet, which proves to be a suitable approach for encapsulating cells and enhancing cell delivery, contributing to its global application. Eighteen variations of 2% (w/v) Low Molecular Weight, alginate hydrogel microcapsules were produced, varying the gas pressure (two levels), syringe pump speed (two levels), and needle diameter (three levels). Statistical analysis using Tukey's multiple comparison test identified the most uniform combination. C2C12 (myoblasts) were microencapsulated within three different alginate groups: 1) non-oxidized alginate, 2) 1% oxidized alginate, 3) 1% oxidized alginate with RGD. Their viability was evaluated using Calcein/7AAD tests every week over 21 days. The released cells during these three weeks were quantified by cell counting. The group using a needle diameter of 21G, a syringe pump speed at 400µL/min and a N_2 Pressure of 50kPa presented the least mean Z-score modulus values for perimeter (1710.4µm ± 110.2), sphericity (0.944 ± 0.019), area (220,712.2µm^2 ± 31,014.3) and diameter (520.6µm ± 32.6), therefore, being the most uniform. These parameters were employed to encapsulate C2C12 cells in the three different alginate groups. By day 21, the cell viability curves for all the groups were reaching a plateau with the following live cells rate: 81.71% (non-oxAlg), 73.25% (1% oxAlg) and 74.92% (1% oxAlg-RGD). Additionally, over the 21 days, the total number of released cells was highest in the 1% oxAlg-RGD with approximately 84,825 cells, compared to 46,300 cells in the non-oxAlg group and 22,975 cells in the 1% oxAlg group. The cell release rate from the microcapsules peaked in the second week for both oxidized alginate groups, whereas the non-oxidized group peaked in the third week. Many parameter combinations for microcapsule production were tested, and the optimal combination was 21G - 400µL/min - 50kPa. The high viability rate of encapsulated cells across all three groups over 21 days indicates a robust cell response. Furthermore, the plateaus in the cell viability curves suggest that the cells successfully adapted to their environment and proliferated. The results also highlight

that the technique effectively supports cell release, which is essential for cell therapy. The variations in release rates among the groups demonstrate that altering the alginate composition can tailor the duration of cell therapy to specific needs, given that all groups maintained high viability rates. Therefore, the microencapsulation technique using the Air-Jet system has shown great potential for advancing cell therapy research globally, being simple, cost-effective, and demonstrating excellent results.

P1023

Genetic Profile of *CFTR* Modulator Ineligible Patients in Turkey

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Although CFTR modulators have been developed to target specific defects in the CFTR protein, these drugs are only suitable for patients with certain variants of CFTR, and eligibility rates vary depending on race and geographical region. This study aimed to reveal the detailed genotype and clinical characteristics of persons with CF (pwCF) at our center in Turkey, a developing country, who are not eligible for CFTR modulators.

As a total, 445 pwCF who admitted to Marmara University were reviewed retrospectively. Variants of the patients ineligible to CFTR modulators were classified based on American College of Medical Genetics guidelines, CFTR classification, the change in the encoded protein, and the variant type.

The study revealed that 139 (31.2%) patients weren't eligible for CFTR modulators. There were 60 different variants in the 276 alleles. The majority of patients had missense or nonsense variants, and that the most common variant was c.1545_1546del, which can be said unique to this geography.

The study highlights the importance of detecting the variants of ineligible patients in detail to guide future approaches for more targeted and effective interventions in CF care. Testing the effectiveness of CFTR modulators for rare or newly occurring variants is crucial to ensure equal access for pwCF to these therapies from different racial backgrounds and ethnic minorities.

P1024

Gene doping detection of seven performance-enhancing transgenes by matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF MS)

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As research and development in gene therapy have continuously expanded in the last decades, such advances have been considered as a risk of potential misuse by athletes to enhance athletic performance and are commonly referred to as gene doping. Gene and cell doping have been prohibited by the World Anti-Doping Agency (WADA) since 2003 and are defined by the alteration of genome sequences or gene expression by the use of nucleic acids, its analogues or genetically modified cells. Its detection in competitive sports is crucial as it not only violates sport ethics but it also poses as a potential health risk for athletes. The Cologne Doping Control Laboratory, in cooperation with Agena Bioscience, has developed a panel prototype for gene doping detection that combines a multiplex polymerase chain reaction (PCR) with single base extensions (SBE) of exon-exon-junction binding oligonucleotide primers and a subsequent SBE-product detection by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis. The panel prototype includes 20 assays for cDNA-detection of seven human transgenes - erythropoietin (EPO), follistatin (FST), growth hormone 1 (GH1), insulin like growth factor 1 (IGF1), myostatin propeptide (MSTN) and vascular endothelial growth factor A (VEGFA) and D (VEGFD) - in one reaction, relevant for gene doping detection in human sports drug testing programmes. Specificity and sensitivity of this panel were examined and all seven transgenes were detectable simultaneously with and without the presence of additional genomic DNA. Furthermore, to test its applicability in routine testing procedures, a mutated sequence (reference material) was designed and validated. Promising results have been obtained so far, giving prominence to this panel as a more time- and cost-effective approach for routine gene doping detection. As potential gene doping preparations are found to be commercially available, this panel prototype was applied to two preparations, and both were found to contain minimal amounts of EPO transgene, therefore underlining its relevance and applicability for anti-doping testing procedures.

P1025

Genetic Assessment of Impurity in Lentiviral vector-based Gene Therapy Products Using Digital droplet-PCR

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The quality control of advanced biopharmaceuticals is crucial for ensuring the safety and quality of final products. Gene therapy products require the management of impurities that may be generated during the manufacturing process. The key concerns in lentiviral vector-based gene therapy are replication-competent lentivirus(RCL) and residual host cell DNA, which can cause a risk of tumorigenicity. For example, reports of the T-cell Malignancy from the patients treated with the CAR-T cell immunotherapies, evoked the risk of using the retroviral vectors, including the 3rd generation lentiviral vectors. Recent MFDS guidelines recommend assessing the possibility of RCL and residual host cell DNA contamination during gene therapy product manufacturing processes. But recent method protocols remain unclear and need to be verified. Even with these ambiguity, assessing vector-related DNA with qPCR were commonly accepted. In the need of developing more sensitive and accurate methods for detecting the minimal amounts of these impurities in gene therapy products, we established the applicable methods for impurity assessment in lentiviral vector-based gene therapy products with digital droplet PCR (ddPCR).

To evaluate impurities of lentivirus vector-based gene therapy products, third-generation lentivirus vectors were produced by transfecting four plasmids (two packaging plasmids, one envelope plasmid and one transfer plasmid) into HEK293T cells. Then we harvested and purified the lentiviral vector to prepare the analytic samples for quality test. VSV-G, known as RCL specific gene and E1A and SV40LTA known as HEK293T specific gene were chosen as markers for ddPCR to detect RCL and residual host DNA. Their primer/probe sequences were designed for method development. Plasmids with each target gene were used as positive controls. Analytical samples were prepared by mixing lentivirus vectors with each positive control plasmid at different concentrations. ddPCR was performed using QX200 system, and following analysis were performed with QX Manager software 2.1.

According to study, plasmids used in the production of lentiviral vectors were characterized by restriction enzyme, PCR, and sequencing to confirm their suitability. The characteristic properties of the lentiviral vector samples were identified by p24 ELISA titer assay. And the absence of RCL specific gene and residual host cell DNA was confirmed by ddPCR. The primers/probes sets were confirmed to detect each target gene under test condition. The broadly used envelope gene VSV-G was targeted to specify RCL, and HEK293T host cell specific E1A and SV40LTA genes were appropriately detected in the sample of lentiviral vector and positive control plasmid, which is transfected in various ratios. Furthermore, the high sensitivity was confirmed by detecting target genes at low concentration in mixed samples.

In this study, we successfully assessed the impurities of lentiviral vector-based gene therapy products by ddPCR. These results can be used as basic data for quality control of advanced biopharmaceuticals. The establishment of these sensitive methods would help assessing not only the viral vectors but also the impurities of the final products for the patients. This research was supported by a grant(24201MFDS199) from the Ministry of Food and Drug Safety in 2024-2025.

Keywords: lentiviral vector, gene therapy, RCL, hcDNA, ddPCR

P1026

Small activating RNAs for treatment of haploinsufficiency in monogenic severe epilepsy

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Dravet syndrome (DS), a severe form of epilepsy, is commonly linked to loss of function mutations in the *SCN1A* gene, which encodes the alpha subunit of the voltage-gated sodium channel Nav1.1. Enhancing *SCN1A* expression presents a promising therapeutic strategy. In this study, we developed 15 small activating RNAs (saRNAs) targeting the *SCN1A* promoter, aiming to upregulate *SCN1A* gene expression to potentially rescue the Dravet syndrome phenotype.

We evaluated the effectiveness of these saRNAs in upregulating mRNA and protein *SCN1A* expression in HeLa and AF22 cell lines using quantitative PCR (qPCR) and Western blot analysis. Furthermore, we assessed the functional impact of *SCN1A* upregulation in a differentiated AF22 neuronal cell culture by measuring neuronal activity through calcium imaging.

Our results indicate that saRNA #10 consistently induced a robust upregulation of *SCN1A* expression across multiple assays. qPCR and Western blot analyses confirmed significant increases in *SCN1A* mRNA and protein levels, respectively. RNA sequencing (RNAseq) further validated these findings, showing a marked increase in *SCN1A* transcript abundance in cells treated with saRNA #10.

In a differentiated AF22 neuronal cell culture, calcium imaging revealed a decrease in neuronal activity upon treatment with saRNA #10. This observation is likely attributable to the cellular composition of the AF22 population, which consists of approximately 40% GABAergic neurons. GABAergic neurons are inhibitory by nature, and their increased activity could counterbalance the excitatory effects anticipated from enhanced *SCN1A* expression.

Overall, our study demonstrates the potential of saRNA #10 as a promising candidate for upregulating *SCN1A* expression, which could serve as a foundational step towards developing therapeutic interventions for Dravet syndrome. Future studies will focus on further validating these findings in vivo and exploring the long-term effects of *SCN1A* upregulation on neuronal function and behavior.

Our findings underscore the potential of saRNA-mediated gene activation as a versatile tool for genetic modulation, with implications for treating a range of genetic disorders beyond Dravet syndrome.

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P1027

Real access to ATMPs: the cost issue

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While industry developed and manufactured Advanced Therapy Medicinal Products (ATMPs) benefit from a centralised marketing authorisation procedure that allows commercialization in all Member States of the European Union (EU), and despite improvement in EU citizen access to ATMPs being stated as one of the two major objectives when the ATMP regulation was released, actual patient access to ATMPs remains one of the most challenging issues to be addressed. Prices of ATMPs are among the highest despite difficulties in proving long term efficacy on significant numbers of patients, although it is fair to acknowledge that cumulative costs of supportive and curative treatments that are administered on repeated occasions may fall in the same ranges. Publicly accessible data show prices that vary from several hundreds of thousands euros to several million euros per treatment, with a continuous trend in increasing prices that has fuelled the concept of “financial toxicity” for those and other categories of treatments. Payment and reimbursement mechanisms as well as decisions on the levels of healthcare expenditures are established at national and possibly in some countries at regional levels by Heathcare Technology Assesment (HTA) agencies and other governmental bodies, with limited possibilities at the European level. Indeed, mainly apart from the Transparency directive and the recently strengthened collaboration in the field of HTA thanks to the adoption of the HTA regulation following the HTA initiatives at the EU level, each Member States is sovereign in determining its health expenses, including to decide on the reimbursement of ATMPs by its National health insurance system. Actual prices paid by public or private payers are not public, and a significant rebate may result from prior and mutual negotiations between drug manufacturers and healthcare providers, the latter receiving reimbursement from health insurers. In addition, specific programs such as the “Early Access” (“Accès précoces”) program in France may speed up access to innovative treatments in some countries, thus favouring a category of EU citizens over others. Thus, actual patient access to ATMPs in the European Union is not only limited, but also heterogeneous, highlighting the existing gap between the intentions stated in the ATMP Regulation and other EU documents and the actual achievements; these diverse factors resulting in an unsatisfactory situation where patients in need of lifesaving treatments face a shortage on a day-to-day basis, not because of insufficient manufacturing capacities as is the case for low-cost drugs, but because of insufficient payment capacities. This poster first provides an overview of the known prices of the 26 ATMPs that have so far obtained a marketing authorisation in the EU. It also highlights what is regulated at the EU and at the National levels in this area. Finally, it suggests potential solutions to improve patients’ access to ATMPs.

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P1028

Clinical Trial Applications for Investigational Medicinal Products that Contain or Consist of Genetically Modified Organisms: Industry Experiences under the European Union Clinical Trial Regulation

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A survey was undertaken Q3 2023, to understand industry experiences when submitting a Clinical Trial Application (CTA) for Investigational Medicinal Products that Contain or Consist of Genetically Modified Organism (GMO-IMPs) since the EU clinical Trial Regulation (CTR; 536/2014) has been in application, that is, since 31st January 2022. EU Member State GMO competent authorities presently apply differing interpretations of the European Commission (EC) Directives for Deliberate Release of GMOs and/or the Contained Use of GMOs. The survey shows how time- and resource-intensive applications seeking authorisations for use of GMO-IMPs to EU Member States continue to represent a significant challenge for developers. Survey feedback highlights how varied the different EU Member State GMO competent authority procedures and assessment timeframes are, with differences in adaptation to the timelines dictated by the CTR. This has led to reported delays to the initiation of clinical trials, especially within the two countries that currently require that the GMO submission be approved prior to submission of the CTA. The benefits of a single clinical trial application submission under the CTR are considerably diminished due to different national GMO procedural and documentation requirements and a lack of formal alignment of timelines between CTA and GMO procedures. There is currently no mechanism for a single submission of GMO applications for multinational trials.

Through the European Commission's reform of the EU General Pharmaceutical Legislation (GPL), instead of needing to submit GMO dossiers to Member State GMO Competent Authorities for each country where it is intended to perform a clinical trial, the EC proposes harmonisation through a centralised, single Union procedure, to be conducted in parallel to a CTA. The European Medicines Agency Committee for Medicinal Products for Human Use (CHMP) shall assess the GMO submission (that includes an Environmental Risk Assessment (ERA) in the form of a Scientific Opinion, for both clinical trials and Marketing Authorisation Applications. The ERA will be tailored to the requirements for investigational medicinal products (instead of GMO plants). The GMO requirements will follow principles similar to that of the Deliberate Release Directive (2001/18/EC). The Contained Use Directive will no longer be applicable and there will be no national notification requirements under this Directive. In April 2024, the European Parliament have voted to adopt amendments to the proposals that include a risk-based approach. This includes the non-requirement for an ERA of investigational medicines with demonstrated low environmental risk, prior to a clinical trial. EFPIA welcome the proposed improvements for regulation of GMO medicines through revision of the EU GPL.

P1029

Nerve conduction assay validation and retrospective analysis of nonclinical safety assessment studies of gene therapies in nonhuman primates

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Nerve conduction studies are key *in vivo* assays for nonclinical evaluation of the peripheral neuropathy liability of gene therapies. Regulatory guidelines describing when and how to include these assays in the safety assessment of drug candidates are not currently established, highlighting the importance of characterizing potential changes induced and hence providing guidelines for inclusion of these endpoints on studies.

A validation study of the nerve conduction assay was conducted at Charles River Lyon by performing repeated recordings of distal sensory (median and sural), motor (peroneal), and proximal mixed (sciatic at the level of the cauda equina) nerves in 3 male and 3 female Cynomolgus monkeys over 5 weeks. Results were reproducible over time and between experimenters. Sensitivity of the assay was tested by manipulating the temperature of the recorded body area and demonstrated that, as described in human studies, nerve conduction velocity (NCV) decreases with decreasing temperature and that this effect is reversible; thus validating the recording technique.

In addition, a retrospective meta-analysis of 14 studies from 5 different Charles River facilities involving gene therapy comprising 11 adeno-associated virus (AAV) vectors, 2 small interfering ribonucleic acids (siRNA), and 1 antisense oligonucleotide (ASO) administered to a total of 418 Cynomolgus or Rhesus monkeys was conducted. The most common administration routes were lumbar intrathecal injection and injection into the cisterna magna. Reduction in NCV or amplitude of evoked responses was observed in 5 studies (36%) and 2 additional studies either showed a trend or were inconclusive due to sample size. Correlation of NCV findings with clinical observations, including neurobehavioral examination when available, and histopathological findings will be discussed.

Overall, this communication will serve to illustrate the nerve conduction technique in nonhuman primates to delineate the best practices for assay design to yield robust and repeatable results in the context of safety/toxicology studies as well as to highlight common gene therapy-associated findings and their clinical and histological correlates.

P1030

A Review of US and EU Expedited Programs for Serious Conditions and Use of the PRIME Toolbox for Expedited ATMP Quality Development in Europe

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The US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) offer programs that are designed to promote agency engagement and expedited development for promising drug candidates. FDA programs include the Breakthrough Therapy Designation (BTD) (2012) and Regenerative Medicine and Advanced Therapy (RMAT) Designation (2016), while the EMA offers the Priority Medicines (PRIME) Scheme (2016). This presentation will provide an analysis of these programs and their utilization, including successes and feedback from industry. During the first few years of BTD, PRIME, and RMAT, it was observed that applicants faced challenges to complete quality and manufacturing development in the timeframe of expedited clinical development. In an effort to alleviate this and to converge on guidance provided to PRIME and BTD products, FDA, EMA, and industry met in 2018 during the "Workshop with stakeholders on support to quality development in early access approaches (i.e. PRIME, Breakthrough Therapies)". During the session, challenges and solutions were explored by real case studies and discussions. For ATMPs in particular, the focus was determined to include comparability, management of out-of-specification products, and the quality development paradigm for autologous products. After additional action from regulators and industry, this workshop resulted in the EMA's 2022 guidance, "Toolbox guidance on scientific elements and regulatory tools to support quality data packages for PRIME and certain marketing authorization applications targeting an unmet medical need" (Toolbox). The Toolbox contains scientific tools that can be applied across manufacturing process and analytical assay validations, adaptive control strategies, and tools for stability and comparability data to support the quality package of a commercial-ready PRIME product. The Toolbox also describes the existing regulatory frameworks that may be leveraged to establish the product-specific acceptability of the scientific tools, as well as the surveillance of their use in the post-marketing phase. The tools will be described in detail in this presentation, using a case study to demonstrate their potential use. In 2023, the EMA and FDA aligned on the use of the Toolbox through consensus Q&A documents encompassing control strategy, process validation, alternatives for determination of re-test period for shelf-life, and GMP considerations (*EMA-FDA joint Q&As on Quality and GMP aspects of PRIME/ Breakthrough therapy applications*, December 2023). As part of this review, we note that this alignment only applies to CDER-regulated products, with CBER-regulated products, such as ATMPs, specifically excluded. This highlights a lack of convergence in the regulation of these products, including those specifically designated for expedited review. This presentation will also review other existing and forthcoming mechanisms that may provide hope for convergence, including the use of parallel scientific advice and FDA's Collaboration on Gene Therapies Global Pilot (CoGent), identified to kick off with a partnership with EMA.

P1031

Addressing the Complex Regulatory Environment for Gene Therapies

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This study explores the evolving landscape of regulatory affairs in gene therapy from Viralgen's perspective, a leading Contract Development and Manufacturing Organization (CDMO) specializing in viral vector production. Here we highlight the complexities of regulatory compliance, the inconsistent guidelines, and the lack of specific regulatory support for gene therapy. We additionally address the complex and dynamic challenges in Chemistry, Manufacturing, and Controls (CMC) support for gene therapy products. Then, we highlight our internal Regulatory team's solutions which include a streamlined production platform process,

comprehensive CMC support, and the implementation of digital solutions such as Regulatory Information Management (RIM) systems and Health Authority feedback databases. We believe the effectiveness of these solutions is demonstrated by our successful regulatory interactions and the approval of numerous clinical trials. The study concludes by discussing future trends in gene therapy regulation, emphasizing faster development pathways, global regulatory alignment, and the necessity for adaptable regulatory approaches to keep pace with scientific advancements.

P1032

Accessible Gene Therapy for Pompe Disease

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Pompe disease or glycogen storage disease type II is an autosomal recessive disease caused by the deficiency of the lysosomal enzyme acid alpha-glucosidase (GAA). GAA is essential for the breakdown of lysosomal glycogen to its glucose monomer; deficiency of this enzyme results in accumulation of glycogen, leading to broad pathology mainly characterized by progressive muscle degeneration and cardiac hypertrophy. Pompe disease additionally presents with neurological pathology. Pompe disease significantly impacts length and quality of life for patients and can result in early death depending on the levels of residual enzyme. The current standard of care is enzyme replacement therapy (ERT) which improves symptoms and lifespan, however it requires expensive (bi)weekly infusions of recombinant GAA, has variable effects across patients, and cannot treat the central nervous system To address these issues, we have developed an improved version of hematopoietic stem and progenitor cell-based lentiviral gene therapy (HSPC-LVGT) as a potential life-long cure for patients following a single intervention. In this strategy the patient's hematopoietic cells are modified ex vivo to introduce an IGF2 tagged version of the missing GAA gene. Through the same cross-correction mechanism exploited by ERT, the modified GAA protein is expressed *in vivo* and can effectively correct the muscular and even brain pathology back to healthy levels via multiple mechanisms, including increased cellular uptake via the cation-independent mannose 6-phosphate/IGF2 receptor. The therapy brings the additional benefit of inducing immune tolerance to GAA, which prevents formation of anti-GAA antibodies. Possible interference from insulin receptor mediated hypoglycaemia was excluded in a mouse model. The success of this gene therapy in preclinical disease models encouraged us to found LentiCure, a non-profit company that aims to develop gene therapy for Pompe disease patients as well as other lysosomal storage diseases at a reasonable and transparent price via a disruptive, non-commercial business model. As the gene therapy employed here concerns platform technology, the LentiCure business model may also serve the future non-commercial development of gene therapies for multiple disorders that are amenable to HSPC-LVGT, including metabolic, immunological, and haematological disorders.

P1033

Next-generation genetic components for expression control in gene therapy

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Current gene therapies often utilize viral or homologous elements that are suboptimal for the intended application. Traditional genetic elements face limitations such as immune responses, undesirable off-target expression, and inefficient gene expression. In contrast, synthetic components can be designed de novo to confer cell-type specificity, high activity, and stability. Here, we exemplify the design of cell-type and product-specific genetic components using a proprietary computational platform that harnesses streams of genomic and proteomic information to identify endogenous regulatory sequences. These elements can be assembled into vector components with user-defined functionality and a minimal DNA footprint, providing a flexible and efficient solution for various therapeutic needs. Targeting the cellular processes of transcription and translocation, we have demonstrated the effectiveness of our technology by developing liver-specific synthetic promoters and Factor VIII-specific signal peptides.

Firstly, we report a series of liver-specific synthetic promoters designed to drive a range of expression in our target tissue, the liver, but only minimal expression in muscle (a critical off-target tissue for liver-targeted gene therapies). The levels of reporter gene expression achieved by our synthetic promoters are comparable to industry-standard benchmark promoters in the model liver cell line, HepG2, yet have significantly lower expression in the differentiated C2C12 skeletal muscle cell line. These synthetic promoters are currently being further evaluated in other model off-target tissues including kidney, cardiomyocytes, and neuronal cell lines, as well as in primary human hepatocytes (PHH) for *in vivo* translatable efficacy.

Additionally, our large, highly product-specific signal peptide library demonstrates the significant impact of signal peptide choice on protein expression. Signal peptides play a crucial role in directing the synthesized protein to its desired cellular location, thereby influencing the overall yield and functionality of the therapeutic protein. When expressing Factor VIII in HepG2, implementation of our signal peptide library leads to higher expression when compared to the native FVIII signal peptide. In doing this, we present signal peptides as an effective and valuable tool for optimising expression levels during gene therapy development.

In summary, we demonstrate that our technologies enhance gene expression control of next-generation therapies, and our diverse range of components allows for a bespoke approach to be applied to any given therapy. When applied effectively, this can lead to an improvement in the safety and efficacy of treatments.

P1034

In vivo viral-free FiCAT programmable gene writing platform validated in Hemophilia A pre-clinical models

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In our previous work we developed a robust gene delivery tool called FiCAT (Pallarès et al; 2021), combining the precision of Cas9 DNA scanning and targeting DNA with an engineered piggyBac transposase with donor DNA processing and transfer capacity. FiCAT allows for programmable and efficient integration of multi-kilobases therapeutic payloads. Here, with the aim to demonstrate FiCAT therapeutic potential in vivo, we performed precise gene delivery to the liver using non-viral carriers that trigger less immunological responses compared to viral-based approaches. Lipid nanoparticles (LNPs) have been extensively validated for the delivery of different nucleic acid cargos. To validate our gene writing platform in vivo, we first optimized both, DNA and RNA LNP formulations. We precisely inserted the DNA transposon codifying our gene of interest by co-delivering FiCAT machinery to mice liver targeting Rosa26 genomic safe harbor. Next, and with the aim of improving our delivery capacity, we optimized different all-in-one formulations to keep all the nucleic acid cargos in the same nanoparticle, screening different lipid mixtures and flow rate. Targeted insertion in vivo was also validated together with high editing levels. Finally, Hemophilia-A pre-clinical models were selected to validate our gene writing technology in vivo. This genetic disorder is caused by disfunction of a large gene (hFVIII, around 9kb) that triggers clotting disorders and hemorrhagic issues. Current ATMP for this indication remain very costly relaying in AAVs that additionally cannot deliver the full FVIII coding sequence. We were able to validate the FVIII expression upon FiCAT mediated integration of the full FVIII CDS and the therapeutic benefits on bleeding tests comparing treated and control models. We also characterized on-target and off-target insertional events, potential immune and toxicity response. Here, we aimed to safely deliver DNA and mRNA cargos via LNPs in vivo thanks to our gene writing machinery (FiCAT). This novel technology opens a window for the treatment of several genetic disorders, including Hemophilia A, making an important breakthrough in gene therapy field.

P1035

Overcoming pre-existing immunity in gene therapy: the HemoSystem DEPLETE approach

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Gene therapy is a promising approach for treating a variety of both acute and chronic diseases. However, pre-existing immunity against viral vectors, such as adeno-associated virus (AAV), compromises therapeutic efficacy and represents a safety concern in gene therapy. For up to 50% of potential patients, gene therapy can be challenging or even impossible, due to pre-existing anti-AAV antibodies. Furthermore, the patients that are receiving gene therapy are at risk for immune-

medicated toxicities. A solution to this problem is to reduce these anti-AAV antibody levels. Current strategies to overcome this problem involve total IgG removal, capsid engineering, or immunosuppression, but these approaches have drawbacks such as affecting the vector performance, biodistribution or transduction efficiency, or can cause adverse events such as infections or organ toxicity. Therefore, there is a need for alternative and more effective methods to modulate the immune response against AAV vectors, improve gene therapy outcomes, and expand the population of treatable patients.

Here, we present a novel medical device-based approach based on an extracorporeal blood purification platform, HemoSystem DEPLETE, which uses magnetic iron-based nanoparticles coated with AAV capsids to remove AAV-specific antibodies selectively and efficiently from the blood. The nanoparticles are injected into an extra-corporeal blood circuit where they bind to anti-AAV antibodies. Then, the nanoparticles along with the bound antibodies are extracted from the circuit using a magnetic filter, leaving the rest of the blood components intact. The cleaned blood is then returned to the patient's body from the external circuit. This way, the antibody levels are reduced and the AAV vectors avoid immune recognition and thus reach the target cells more efficiently.

We conducted a feasibility study to demonstrate the synthesis of the nanoparticles coated with AAV9 capsids and the removal of anti-AAV9 antibodies *in vitro*. The results showed that the nanoparticles could be successfully synthesized and covalently functionalized with AAV9 capsids. AAV9-coated nanoparticles were applied to human plasma samples from six donors with different total antibody binding (TAb) anti-AAV9 titers, ranging between 1:27 – 1:243. The nanoparticle treatment reduced the TAb titer to 1:3 – 1:9, well within the dosing range of most current commercial AAV therapies. Neutralizing antibody (NAb) titers were reduced from an initial NAb titer ranging between 1:243 – 1:177'149 to 1:9 – 1:27 after nanoparticle treatment. Moreover, we demonstrated that the AAV9-coated nanoparticles maintain their anti-AAV9 antibody-binding capability in whole blood and also that nanoparticles equipped with empty AAV9 capsids were equally effective as nanoparticles coated with full capsids.

In conclusion, HemoSystem DEPLETE is a feasible and innovative solution to overcome the pre-existing immune response against AAV vectors, expand patient access and improve gene therapy outcomes. This technology could potentially enable the repeat administration of AAV vectors, expand the patient population eligible for gene therapy, and improve the clinical efficacy and safety of gene therapy for a variety of genetic diseases.

P1036

Awareness, perception and knowledge of gene therapy among the general public vs. chronically ill patients in Austria

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Gene therapy is a new and evolving treatment option for various genetic disorders entering the clinical routine. Therefore, we aimed to understand the knowledge and perception surrounding the topic of gene therapy in the community. From November until December 2023, we

conducted an online survey among 119 adult patients recruited from a panel of patients with chronic disease and 506 persons aged >18 years from a representative sample of the general population in Austria, Europe. Of the 625 participants, 56% considered themselves as healthy (H), 44% suffered from chronic diseases (CD) with mild (37%) or severe health impact (7%), and 7% had an inherited disease (ID). More than half of the respondents (58%) had already heard of gene therapy. Among these, 64% expressed an interest in gene therapy (64%H, 56%CD, 87%ID) of which 47% (45%H, 49%CD, 68%ID) had already actively gathered information about the topic. Physicians were the primary source of information (75% all, 69%H, 82%CD, 81%ID), followed by the internet (72%) and medical newspapers, scientific journals or books (32%). When asked to explain gene therapy, the answers were mainly neutral (41%H, 40%D, 54% ID), 7% were negative (6%H, 8%CD, 14% ID) and 5% positive (5%H, 4%CD vs 9%ID). Participants were also asked to evaluate predefined statements about gene therapy, with 67% considering gene therapy as a big chance with a potential to cure diseases, 70% agreed that gene therapies are used to treat genetic diseases by replacing or repairing genes and 51% responded that the long-term effects are still unpredictable. In terms of ethical considerations, 47% of individuals with IDs found gene therapy ethically problematic compared to 29% of healthy individuals. Overall, people with IDs tended to agree more often with the predefined statements, however, also with the statement, that viruses used in gene therapies can still transmit infections. Healthy and diseased individuals associated similar levels of hope (62%H, 57%CD, 68%ID) and confidence (54%H, 52%CD, 62%ID) with gene therapy. There was a slightly higher safety concern in IDs vs. the entire survey population (44% vs 31%). The most desired outcome of gene therapy was cure (74%), followed by halt in disease progression (66%), slowing down of progression (52%), reduction of symptoms (49%) and absence of symptoms for a few years (40%), with the exception that individuals with IDs ranked the slowing of the disease progression as the second most desired outcome. Interestingly, in case of a disease, 70% of all participants claimed that they would undergo gene therapy, if available. The survey also revealed differences based on educational background, region, and age, but not gender.

In conclusion, this survey demonstrates that the majority of people in Austria had heard of gene therapy, perceives it as a significant opportunity and associates it with hope. Minor differences were seen in the healthy vs. the diseased population, but notable differences were found when comparing to patients with IDs. As physicians remain the primary source of medical information, their knowledge plays a crucial role in shaping public understanding of gene therapy.

P1037

Addressing sustainability of ATMPs by innovative platform approaches

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Advanced Therapy Medicinal Products (ATMPs) define a broad category of complex and innovative therapies which encompasses tissue, cell-based products, and gene therapy. These revolutionary medicines offer groundbreaking new opportunities, addressing unmet medical needs for rare and complex diseases.

However, numerous challenges persist. Among these, high costs and resource demand pose significant hurdles especially during the transition from bench to bedside, when the requirement for GMP compliance enters the game. In this context, fostering a shift toward a comprehensive

platform approach, that encompasses both analytical and manufacturing endeavors, emerges as an essential strategy to enhance the sustainability and affordability of ATMPs.

A platform consists in an analytical or manufacturing solution which is applicable to multiple different products that share a pre-defined degree of similarity, without significant changes in its operational conditions. Of note, FDA further elaborated the concept of platform in its recent draft guidance published May 2024, possibly broadening its application to the product itself, with a potential game-changer impact for the ATMP field.

Here we will present the way we are interpreting the platform approach, the preliminary results of our activity, and the related advantages: by consolidating set up and qualification efforts into analytical and manufacturing platforms applicable across multiple drug categories, significant reductions in overall resource needs are possible. Advantages include reduced overall costs, shortened timelines and reduced sample volumes needed, while ensuring the availability of validated analytical methods and robust manufacturing processes from the earliest stages of drug development. This will help overcoming constraints related to resource allocation and production scale that often hinder such level of robustness in the preclinical / early clinical research.

P1038

Patient identification: is there a need for an early diagnosis strategy for rare disease gene treatment?

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There are over 7000 rare disorders, but therapies are only available for approx. 5 % of them. Regardless of available treatments, the set-up for a particular disease is country-specific and driven by diagnosis and treatment strategy. Its changes are mostly center-driven, with limited systematic reach, not automatically allowing to reflect the accelerated developments of innovative therapies. In the rare disease portfolio, advanced therapies such as cell & gene treatment (CGT) have been added recently during the last decade. It is therefore not surprising that doctors are not always updated on available treatment options. Moreover, newborn screening (NBS) is not implemented for all rare diseases with causal treatment available, and healthcare systems are not always set up to pursue full differential diagnosis. Without an early diagnosis strategy, patient identification remains a crucial obstacle in access to advanced treatments.

Since early diagnosis matters when causal treatment is available, we investigated whether Slovak doctors are provided with accessible tools for specific and rare diagnoses.

We interviewed 26 pediatric neurologists in Slovakia (representing over 40 % of outpatient clinics in the country) about their diagnostic approach to hereditary demyelinating disease of the central nervous system.

During the last 24 months, only one specialist tested for the disease over 5 times, 5 specialists tested once or twice, and 20 specialists did not perform any test. They stated that during diagnosis they proceeded from most frequent to less frequent diseases and that they specifically tested for the disease after more symptoms were present, to confirm diagnosis rather than to rule it out.

The outcome of the survey reflects the current diagnostic setup, which is not allowing early access and full effects of CGT. The confirmation of the diagnosis of this rare disease is done usually in later stages with manifesting symptoms, since it is tested only after more symptoms are present, thus often missing the therapeutic window. It is debatable if increasing testing of high-risk populations with less specific symptoms could aid diagnose patients at earlier stage, meeting criteria for gene therapy. Until NBS is implemented for treatable rare disorders and automatic referral to specialized treatment centers is established, more frequent testing for treatable rare diseases should be assessed. In case of newly available causal treatment, the objective should be to develop a system which reflects recent therapeutic developments in a timely manner, to allow early diagnosis and better treatment outcomes by early treatment initiation.

P1039

Selection of qualified treatment centrum for cell therapy

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The number of small companies entering the field of cell & gene treatment (CGT) is increasing significantly (currently 47 %). These new start-ups lack a history of promoting their products, are often not present in most countries and realize limited launch activities to introduce new treatments. Cell therapy Holoclar was developed by such a smaller company and was approved in EU for use in adult patients with moderate-to-severe limbal stem-cell deficiency caused by trauma (burns, including chemical burns to the eyes). Patients often lose sight on the affected eye. The Holoclar treatment can be performed only in a qualified treatment center (QTC) under EMA regulations. It is critical to select a center that will qualify and will cover a significant part of the population. It is challenging to set up an effective patient flow from diagnosis to treatment and select treatment centers that would undergo the tedious process of qualification and certification. Often these centers do not have established processes for referencing patients from other regions and centers. Mostly the patients are referenced based on personal requests of attending doctors with established personal contact with the doctor from the QTC.

We mapped the existing practice to help to understand where the patients were concentrated, and whether the patients were treated at primary contact or referenced to specialized centers. The following criteria were considered: number of patients suffering from disease in the population, number of patients treated in the center, expertise in disease in the center, interest in topics by team and KOL involved, specialization in disease area, and team size with specialization. Other aspects were also considered: established process for treatment, equipment, skills, qualification, size coverage of region/ country, support of new activities by center/hospital management including matrix support like legal and finance department delivery, tissue lab.

From 10 centers we narrowed down the selection to 3 centers: Prague VFN, Prague Vinohrady, FN Brno. Despite the smaller number of patients connected with VF Brno (in contrast to Prague centers), VF Brno was finally selected for completion of qualification, based on the fact that it was the furthest in the qualification assessment process, readiness to complete agreements and legal & financial documents. In VF Brno the hospital management was the most supportive, showed interest and matrix support.

Since most patients were located and treated in Prague, we proposed and successfully arranged a novel approach - a patient-doctor tandem to travel from Prague center to Brno qualified Holoclar center.

When selecting a center for qualification, it is critical to evaluate the current treatment skills of the center as well as patients' pathways and accessibility to a bigger population. The effective management of the center and its supportive functions to address legal, financial and qualitative aspects is mandatory. The flexibility to find solutions during challenging times of COVID or team changes (maternity leave, sickness, relocation) is required to maintain accessibility to treatment. Management of more complex processes and a broader team was effectively managed and coordinated by the support team, Medasol.

P1040

Engineered Mesenchymal Stem Cells for *ex vivo* culture of human Hematopoietic Stem and Progenitor cells

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Mesenchymal Stem Cells (MSCs) have proven to be an exceptional co-culture platform for Hematopoietic Stem and Progenitor Cells (HSPCs), significantly enhancing their expansion and preservation of stemness (1). Additionally, MSCs facilitate better recovery post-gene manipulation and improve the engraftment efficiency of gene-modified HSPCs(2). However, this system also requires the supplementation of exogenous cytokines to support HSPC culture which elevate the costs of HSPC cultures in both pre-clinical and clinical settings. To address the challenge and to have a simplified platform, we have developed an innovative co-culture system in which MSCs are genetically modified to secrete critical growth factors, including SCF, FLT3, TPO, IL3, and TXN. In our study, we compared gene-modified bone marrow-derived MSCs (GM-BM MSCs) and Wharton jelly-derived MSCs (GM-WJ MSCs) with the standard culture conditions, where HSPCs are cultured in the presence of exogenous cytokines without MSC support. Over a six-day culture period, our results demonstrated a significant increase in the absolute numbers of $CD34^+$ $CD90^+$ cells in both GM-BM MSC and GM-WJ MSC co-cultures compared to the standard conditions. Moreover, culturing under physiological oxygen levels (5% O2) and in the presence of RUS (Resveratrol, SR1, and UM729) cocktail further enhanced the proportion of $CD34^+$ $CD90^+$ cells *in vitro*. Consequently, we observed a substantial rise in the absolute number of more primitive HSPC populations ($CD34^+$ $CD90^+$ $CD38^-$ $CD45RA^-$ $CD49f^+$ cells) in the MSC co-cultures. Importantly, gene editing of HSPCs for a homologous directed repair (HDR) approach showed comparable editing frequency between the different culture conditions. Our findings reveal a marked enhancement in the retention of HSPC stemness within the engineered MSC co-culture system. This validates the efficacy of MSC-mediated growth factor delivery as a viable and promising strategy for optimizing HSPC culture. The approach holds significant potential for advancing therapeutic applications in hematopoietic regeneration and gene therapy, offering a cost-effective and efficient alternative to traditional cytokine supplementation. These results underscore the transformative potential of MSC-based growth factor delivery systems, paving the way for more effective and economically viable strategies in stem cell research and clinical applications.

P1042

JOIN4ATMP Consortium: New frameworks for value assessment, pricing and reimbursement schemes of Advanced Therapy Medicinal Products

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Advanced Therapy Medicinal Products (ATMPs) are medicines based on genes, cells and tissues for human use that can sustainably improve or even cure diseases that currently have no or inadequate standard-of-care options. However, several hurdles remain unsolved and prevent the implementation of these innovative therapy options into effective clinical application. JOIN4ATMP Consortium aims to accelerate European ATMP development and ensure wide-spread ATMP access. To achieve this ambitious goal, the project will focus on three processes: categorizing obstacles to ATMP progress, mapping potential solutions based on real-world use cases and designing actionable recommendations. Bringing together specialists, physicians and researchers from fourteen partners based in Europe, JOIN4ATMP is coordinated by Charité - Universitätsmedizin Berlin supported by Berlin Institute of Health (BIH), and co-coordinated by Università Vita Salute San Raffaele, and will be funded with EUR 3 million from the European Union's Horizon Europe Framework Programme - Coordination and support action (CSA) over the next three years. JOIN4ATMP joins all members of the European University Hospital Alliance (EUHA), the existing EU-funded T2EVOLVE and RESTORE networks, Fraunhofer Gesellschaft, the European Organisation for Rare Disease Association (EURORDIS) and EURICE. This collaborative effort offers a privileged and diverse long-standing expertise in the central ATMP application fields: rare cancers, rare non-cancers including rare genetic diseases, and common diseases. Within this project, Università Vita Salute San Raffaele will lead Work Package 4 (WP4) with the support of Fondazione Telethon. WP4 aim will be to use real-world data to map hurdles in the commercial uptake of ATMPs, including pricing and reimbursement challenges. We will propose how ATMP developers could improve their interactions with health technology assessment (HTA) bodies throughout ATMP development, improve usability of real-world data for HTA evaluation, and propose alternative pricing and reimbursement schemes. JOIN4ATMP will reach out to stakeholders in the field to actively engage them via semi-structured interviews and questionnaires in the mapping of hurdles, charting of solutions, and design of recommendations for a streamlined implementation of ATMPs developed by academia, pharma, SMEs or not-for-profit organizations.

P1043

From bench to dose: building a robust and scalable cGMP manufacturing process for non-replicative Herpes Simplex Virus 1 (nrHSV-1) vector as a powerful gene therapy platform

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Viral vectors have become one of the leading delivery systems for genetic medicine in humans. The specific combination of their key properties offers several advantages tailored to a broad spectrum of gene therapies for both acute and chronic diseases. nrHSV-1 vectors are attractive for gene therapy mainly due to the large HSV genome (152 kb) which can accommodate therapeutic transgenes larger than 30 kb in size. Moreover, they are particularly useful in nervous system (NS) gene therapy applications due to their innate neural tropism and ability to establish latent infections with prolonged transgene expression. Many of these indications address large patient populations necessitating efficient, large-scale production to reach scale and lower COGS per dose. Thus, developing a highly productive, scalable and completely closed production process represents a critical requirement to manufacture the vector under cGMP conditions to cover the clinical demands. This study describes the development of a fully scalable production process for a nrHSV-1 viral vector using the Fixed-bed Scale-X technology from the bench up to the Scale X Carbo 30 m^2, using a derivative clone from an adherent Vero cell line. The upstream and downstream process parameters were initially investigated by small-scale studies using a Cell Stack system, then the optimal parameters were adapted and transferred to the Scale-X Hydro 2.4m^2. A number of optimization runs were executed to increase productivity and maximize the yield and clearance of the final process. By infecting the cells at a density of >1.2E+05 cell/cm^2, a productivity comparable to the values obtained in Cell Stack systems was achieved. In terms of downstream, the release of the virus from infected cells was coupled to an endonuclease digestion in recirculation mode inside the bioreactor, ensuring an efficient system to recover the intracellular virus, while reducing the host cell DNA released from the cells. Furthermore, a 0.45 µm clarification step was introduced to reduce cell debris and aggregated impurities, while the TFF with hollow fiber was carried out to formulate the final DS. Our modular purification platform ensured optimal overall yield (≥50% in terms of PFU/mL) and good impurities clearance (up to 785-fold HCP reduction and HCDNA amount ≤10 ng/dose) in final product. To prove the full scalability of the process, additional runs were executed on both Scale-X Carbo 10m^2 and Carbo 30m^2 bioreactors, as demonstration and engineering runs respectively, to mimic the final process implemented during GMP manufacturing. The process parameters were linearly scaled-up based on the higher fixed bed surface, while the same seeding and infection cell densities were applied. Although a higher fixed bed scale was used, both vector-specific productivity (pfu/cm^2) and process yield (~ 50%) were achieved. The manufacturing of the clinical lot was successfully executed, proving the development of a robust and scalable platform fulfilling clinical dose expectations and satisfying regulatory requirements for the release of the drug product.

P1044

Autonomous synthetic cells for the detection and counteraction of bacterial infections

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Genetically modified cell therapy gained momentum in the context of various diseases with great success. However, host cell-based therapies that interfere directly with bacterial infections are rare. While most bacterial infections can be treated with existing antibiotics, rising antibiotic resistance is a common but critical issue in healthcare settings, contributing to significant mortality and morbidity each year around the world. The stagnation in the development of novel antibiotics also worsens the problem. In addition, the diagnosis of bacterial infection is often possible once patients present with obvious symptoms, making the therapy even more challenging. In this study, we aimed to design and explore a novel cell-based system that diagnoses bacterial infections at an early stage and automatically produces therapeutic proteins. Our strategy employs genetically engineered sensor/actor cells that can sense type I interferons (IFNs), released by immune cells during their interaction with pathogens. To this end, we developed an infection-responsive intracellular synthetic network by integrating the synthetic trans activator tTA into the locus of the endogenous Mx2 gene, which is activated by type-I/III IFNs. This Mx2-tTA module was rewired to P_{tet} promoter-controlled immune-stimulatory factors and reporter genes. A positive feedback module was implemented to achieve long-lasting activation of the system after the initial IFN stimulation, which is controllable by the addition of doxycycline. To mimic physiological scenarios, co-cultures of the infection-responsive sensor-actor cells and macrophages were exposed to bacteria. Both gram-positive and gram-negative bacteria induced the synthetic signalling cascade, enabling the sensitive visualisation of infections in a dose-dependent manner. Implementing the positive feedback module P_{tet}-tTa induced a response that was maintained even if the trigger had faded out, while doxycycline treatment reset the sustained expression to the basal level. The positive feedback module also allowed overriding cellular negative feedback mechanism of the endogenous IFN pathway that fine-tunes IFN activity during sustained acute and chronic inflammatory situations. We demonstrated that this infection-induced synthetic cascade can be harnessed to secrete immunostimulants like CCL2 and CCL22. In vitro, these factors enhanced immune infiltration, which has the potential to improve bacterial clearance. In conclusion, our autonomously controlled cellular system is characterised by high sensitivity and can potentially combat bacterial infections in various scenarios (e.g. implant surfaces). Furthermore, this concept may be applied to the development of cell-based diagnostic and therapeutic alternatives relating to different inflammatory diseases.

P1045

GMP-Compliant iPS Cell Lines Show Efficient Cardiac Induction in a New Differentiation Platform Featuring Scalability and Enhanced Robustness

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Induced pluripotent stem cell (iPSC)-based therapies hold significant promise for treating various diseases, including heart failure. However, the implementation of such therapies faces challenges due to the stringent quality and consistency requirements imposed by Good Manufacturing Practice (GMP) regulations. To address these challenges, Catalent has established a GMP-compliant workflow for generating iPSCs using episomal reprogramming from eligible donors in the EU and US. The resulting iPSC lines met comprehensive release specifications, showed no critical cancer-associated gene lesions, and exhibited a low global mutation load, indicative of their neonatal origin from cord blood. Beyond the generation of this critical starting material, Catalent has been developing novel and improved manipulation platforms to facilitate translation to a GMP setting in a partnering model. These include an optimized gene editing methodology and advanced and GMP-compatible differentiation protocols for retinal pigment epithelial (RPE) cells, mesenchymal stem cells (MSCs), as well as immune cells such as natural killer (NK) cells.

Finally, we have developed a GMP-compatible workflow for converting HLA-homozygous iPSCs into cardiomyocytes (CMs). Applying a co-stimulation strategy involving both WNT and BMP signaling to induce cardiac mesoderm resulted in improved differentiation efficiencies. However, true robustness of this methodology was achieved only upon extending the co-stimulation strategy to the FGF and TGFβ signaling pathways, in conjunction with specific adjustments to media changes. The resulting CMs initially exhibited an immature gene expression profile but matured over time, acquiring a pronounced sarcomeric structure and an overall ventricular fate. Notably, this revised methodology effectively eliminated bulk cell density as a critical process parameter and significantly increased consistency. This improvement allows for multiple high-efficiency differentiation runs to be performed consecutively – a key pre-requisite for GMP translation. The workflow is scalable to larger culture volumes and compatible with a prior expansion of the iPSC using an independent 3D system, thus enabling a combined therapeutic platform.

In summary, these GMP-compliant iPSC lines and compatible manipulation platforms solve critical bottlenecks in iPSC-based cell therapy, which will enable a facilitated path to clinic for those key indications.

P1046

Flow cytometry as a tool for potency determination in ATMP development

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Developing methods to determine potency for Advanced Therapeutic Medicinal Products (ATMPs) is particularly challenging. The assay(s) must strike a balance between science, compliance, and efficiency. Flow cytometry has shown significant potential as a platform for potency assays due to its selectivity, multiple potential readouts, and ability to establish a functional relationship to the therapeutic mechanism of action. There are specific situations in which the application of flow cytometry may offer discrete advantages. In this effort, we share several case studies to demonstrate the application of flow cytometry to the phase appropriate development and/or validation of potency assays for ATMPs including gene therapies, lipid nanoparticles, mRNA, and other advanced drug modalities. The detection targets include replacement proteins, surface expressed antigens, and others. These applications demonstrate selection of the appropriate technique, comparison of flow cytometry platforms, considerations

for the transfer of methods between labs or platforms, and the transition from other cell-based techniques to flow cytometry. The impact of laboratory conditions, analyst-to-analyst variability, critical reagents, and platform differences on the performance of the assays is presented. The significance of gating and data processing approaches is also addressed, with strategies for optimizing these methods. The suggestion for establishment of phase appropriate control parameters – a.k.a. assay acceptance or performance criteria – is included. Case studies compare flow cytometry with other detection approaches, providing insights into when to use or avoid this technique for potency assays. Finally, the creation of criteria for the qualification, validation and/or transfer of flow methods is discussed to facilitate the implementation of these methods as part of robust potency assurance strategy.