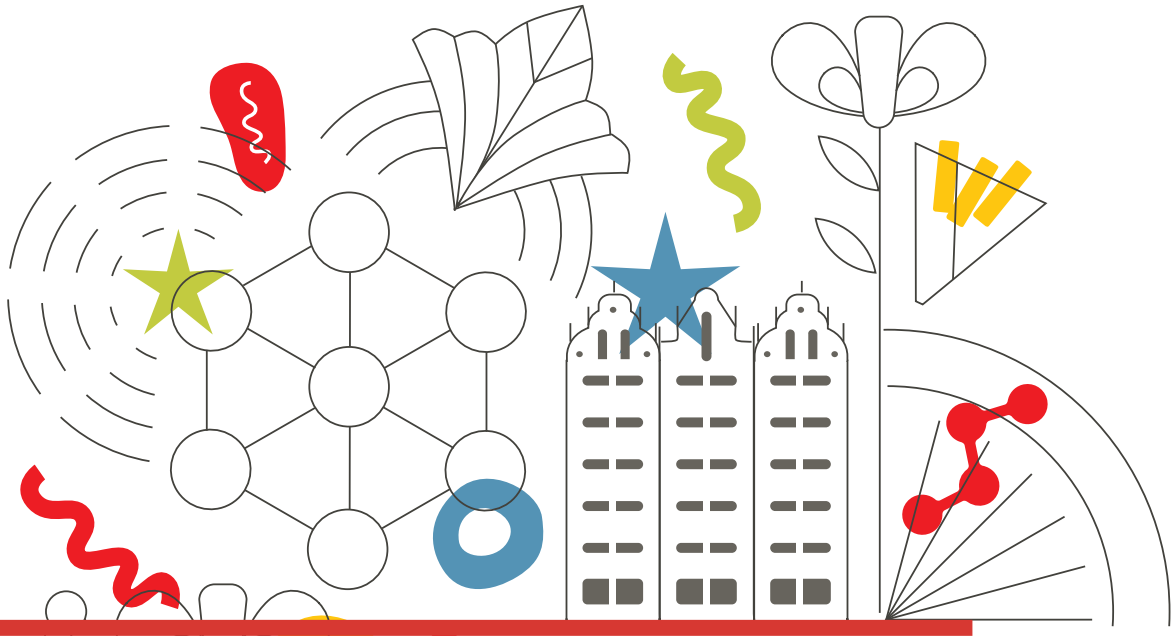

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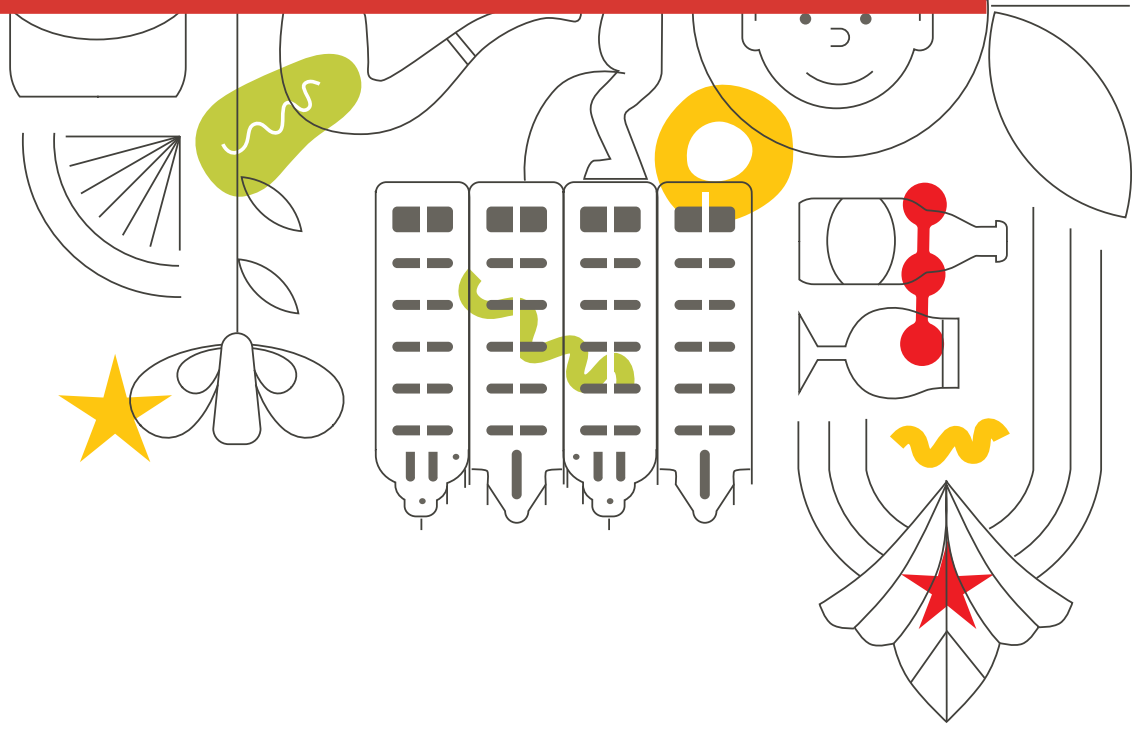

EUROPEAN SOCIETY OF GENE & CELL THERAPY

Société Française de Thérapie Cellulaire et Génique SFTCG

NVGCT Nederlandse Vereniging voor Gen- & Celtherapie

Invited Speakers

EDU02

Adeno-associated virus (AAV) vectors - from natural occurring serotypes towards engineered nanoparticles

H Büning [1]
1: Hannover Medical School

Adeno-associated viruses (AAV) vectors are the most widely used delivery tools for in vivo gene therapy. As market approved gene therapy medicinal products (GTMPs) AAV vectors are applied either locally or intravenously for the treatment of monogenic diseases. Monogenic diseases related to liver, eye, the central nervous system, or muscle are also in the focus of an impressive number of ongoing AAV vector-based human clinical trials. Moreover, in recent years, application of AAV vectors has been expanded towards the areas of oncology, infection diseases and vaccine development. Despite this success, usage of AAV vectors derived from natural AAV serotypes revealed several limitations: 1) prevalence of pre-existing neutralizing antibodies due to exposure to the wild-type virus, 2) de novo induction of immune responses, 3) high vector doses that are required to overcome pre- and post-entry barriers towards AAV vector-mediated transductions and 4) loss of vector particles in off-target tissues.

In response to these challenges next generation AAV vectors are developed delivering improved vector genomes within tailored capsids. Moreover, in-depth characterization of the vector-host interaction allowed to streamline the use of AAV vectors and to improve vector efficacy by targeted manipulation of key limiting steps in cell transduction. On this background, the lecture will briefly introduce the AAV vector system, followed by presenting examples of strategies applied to overcome the mentioned key challenges to empower the AAV vector system.

EDU07

CRISPR-Cas technology - a toolbox that is changing research and medicine

J Grünewald [1 3]
1: Technische Universität München 2: 3: Klinikum rechts der Isar der TUM

CRISPR technologies have had a transformative impact on research and they have enabled the development of new therapies in a relatively short period of time. The suite of CRISPR-based gene and epigenetic editing tools is constantly expanding and a large variety of modalities can now be customized to specific use cases. This educational talk will give you an overview of the newest CRISPR technologies, applications, and the clinical translation into new gene and cell therapies.

EDU09

Advanced disease modelling for neuromuscular genetic therapies: iPSCs, 3D cultures and beyond

F S Tedesco [1]
1: University College London

Skeletal muscle is a complex tissue composed of multinucleated myofibres supported by a variety of cell types and a specialised extracellular matrix (ECM), compromised in severe incurable neuromuscular diseases such as muscular dystrophies (>40 disease-causing genes identified to date). Preclinical work and recent positive clinical trial outcomes position gene therapy as a key experimental treatment strategy for several genetic disorders, including spinal muscular atrophy (SMA). However, despite several years of intense preclinical and clinical work, only one gene therapy has recently received accelerated FDA approval for a form of muscular dystrophy (and no genome editing strategy is approved for any muscular dystrophy or other primary skeletal muscle diseases). Limitations in animal models (e.g., ethical, financial and biological) as well as lack of reliable, human(ised) in vitro models currently pose hurdles towards development of novel neuromuscular therapies. To address these limitations, there has been a growing interest in recent years to harness the potential of emerging technologies such as human induced pluripotent stem cells and organoids, coupled with new scaffolds and biomaterials. I will therefore review progress in developing advanced human skeletal muscle platforms to model different morphological and functional defects in tissue compartments impaired in neuromuscular disorders (e.g., sarcolemma, nuclear envelope, ECM). I will also discuss their application to screen and validate experimental therapies, focusing on their potential to facilitate development of novel gene therapy and genome editing strategies for neuromuscular disorders.

EDU11

Recent developments in muscle-directed gene therapy

F Mavilio [1]
1: Università di Modena e Reggio Emilia

Systemic administration of muscle-tropic adeno-associated viral (AAV) vectors is a clinically validated strategy to deliver genes or gene editing complexes to skeletal and heart muscle in patients affected by inherited muscle diseases. Recent clinical trials have shown therapeutic efficacy but also significant systemic and organ toxicity. To target the muscle, AAV vectors are delivered intravenously at very high doses (>104 vector genomes per kg of body weight). Since currently used AAV serotypes are mostly captured by the liver, high doses result in significant liver toxicity and in some cases severe and potentially lethal triggering of innate immune responses. We will discuss the risk and benefit of muscle-directed gene therapy, taking Duchenne muscular dystrophy and myotubular myopathy as examples.

EDU12

Recent developments in liver-directed gene therapy

P Bosma [1]
1: Amsterdam UMC

The liver is an attractive target for gene therapy. Not only because of its central role in many metabolic processes and detoxification but also because of its capacity to produce and secrete proteins. In addition to endogenous factors like albumin and clothing factors, this can capacity render the liver suitable to compensate deficiency of for instance lysosomal enzymes in other tissues.

Different gene therapy strategies and vectors have been investigated. Especially gene addition strategies using Adeno Associated Viral Vectors and proved to be safe and effective, resulting in market approval for applications in adult patients. For treatment of children, patients with antibodies towards the AAV vectors and disorders causing liver damage important hurdles need to be overcome. Currently, several options, like gene repair and/or the use of other vectors, are investigated to make gene therapy available to all patients suffering from an inherited liver disorder. The (dis) advantages and these novel strategies will be discussed.

INV02

Hemophilia gene therapy: advances and challenges

M C Ozelo [1]
1: University of Campinas, Hemocentro UNICAMP, Campinas, SP Brazil

Hemophilia, an inherited bleeding disorder resulting from deficiencies in clotting factors, has historically presented significant challenges in its management. Recent advancements in gene therapy have opened promising avenues for treating hemophilia to provide patients with a lasting solution. This abstract offers an overview of the current state of hemophilia gene therapy, highlighting both its advances and challenges.

Gene therapy for hemophilia has made remarkable progress, driven by the development of adeno-associated viral vectors (AAV) as highly efficient delivery vehicles for clotting factor genes. These vectors have demonstrated impressive safety and efficacy profiles in clinical trials. Successful gene therapy interventions have translated into improvements in clinical outcomes, notably reducing the frequency of bleeding episodes and enhancing the quality of life for patients. This has resulted in a reduced burden of frequent factor infusions.

Notably, two gene therapy products have recently obtained approvals from the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA). These products comprise Rocatavian (valoctocogene roxaparvovec), an AAV5 vector expressing factor VIII designed for adults with hemophilia A, and Hemgenix (etranacogene dezaparvovec), an AAV5 vector expressing the factor IX-Padua for the treatment of adults with hemophilia B.

However, numerous challenges persist in the field of hemophilia gene therapy. A noteworthy issue in all AAV-based clinical trials has been the high variability observed in factor VIII and factor

IX expression levels. This variability has led to unpredictable responses in both hemophilia A and B gene therapy thus far. Additionally, the immunogenicity associated with the AAV vector has emerged as a significant hurdle in the follow-up care of these patients. This necessitates the administration of prolonged and often high-dose immunosuppressive regimens, particularly during the first year following gene therapy.

Furthermore, concerns have arisen regarding the durability of factor VIII expression, with evidence of declining levels in hemophilia A gene therapy over time. This decline raises apprehensions about this strategy's long-term efficacy and sustainability for hemophilia A.

Lastly, addressing safety concerns is paramount. Ensuring the prolonged safety of gene therapy interventions, including the potential risk of insertional mutagenesis, remains a top priority that demands vigilant and long-term monitoring.

In conclusion, hemophilia gene therapy has witnessed remarkable advancements, offering hope for a paradigm shift in the treatment of this rare bleeding disorder. While significant strides have been made in addressing challenges related to safety and efficacy, ongoing research and collaboration are essential to overcome the remaining hurdles. The ultimate goal is to make gene therapy accessible to all patients with hemophilia, improving their overall quality of life and reducing the burden of this lifelong condition.

INV03

Successful clinical use of gene addition and transcript knockdown in SCID-X1 and Sickle Cell Disease

D Williams [1]
1: Boston Children's Hospital, Dana-Farber Cancer Institute, Harvard Medical School

We have utilized distinct viral vector approaches to modify two serious diseases with success. Severe Combined Immunodeficiency-1 (SCID-X1) is a fatal disease caused by mutations in IL2RG, which encodes the common gamma chain (γc) required for function of multiple cytokine receptors. Based on the occurrence of insertional mutagenesis in previous trials and iterative vector designs, we developed and implemented into the clinic a LV vector backbone using the same EFS promoter that was successful in reconstituting T cell immunity in our previous SIN-γRV trial (Hacein-Bey-Abina *NEJM*, 2014) expressing the IL2RG but with no insulator sequences and hypothesized this vector would be associated with continued efficacy and an enhanced safety profile with respect to insertional oncogenesis, as manifested by a lack of overt leukaemia and lack of sustained expansion of dominant clones. This is an international trial including multiple centers (NCT03311503; NCT03601286) with sites in GOSH, Boston Childrens, CHOA, CCHMC and UCLA.

We have data of the first 14 patients who underwent gene therapy in this study. Reconstitution of T cells was robust and uniform with all patients. Humoral immune reconstitution was consistently seen. Of 13 patients with ≥6 months of follow-up, 11 have discontinued IgRT at a median of 12 months (6-14.6) post-infusion. No adverse events related to the medicinal product have been reported. Insertion Site Analysis (ISA) showed a polyclonal reconstitution and a distribution pattern typical for LVV with no expanded clones with insertions in *LMO2* or *HMGA2* detected. The maximum extent of expansion of clones with *LMO2* or *HMGA2* integrations was <1% to date. In contrast to the approach of addition of a cDNA to express a normal protein, a dramatically different approach is to express a shRNA

embedded in a microRNA (termed a shmiR) (Brendel *JCI*, 2016) to knockdown expression of a repressor protein, in this case BCL11A, the major repressor of γ-globin expression in adult red blood cells, leading to reactivation of fetal hemoglobin (HbF) expression as a treatment for β-hemoglobinopathies. High expression of HbF mitigates intracellular polymerization of deoxygenated sickle hemoglobin (HbS). By targeting BCL11A, we reverse a intracellular physiological switch and simultaneously and concurrently reduce HbS while increasing HbF. We have embedded a BCL11A shmiR into a LVV. Because of the shmiR configuration and cis-regulatory elements in the vector, the knockdown is restricted to erythroid cells, avoiding on-target toxicity in B lymphocytes and HSCs. The approach has been successfully employed in a pilot study (Esrick et al., NEJM, 2021) and is the basis for a multi-center phase 2 pivotal study in the US (NCT05353647). To date, there have been no toxicities related to the vector and engraftment of patients in the pilot study (N=10) shows no notable expanded clones or clonal skewing.

Based on this success, we have collaboratively (D. Kohn, UCLA) developed a bifunctional LVV using the shmiR technology with the capacity of simultaneously knocking down multiple genes in tandem while concurrently expressing a cDNA. The capacity to modify multiple gene functions simultaneously expands the uses of LVV in treating clinical diseases

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INV04

Exploration of biological diversity

F Zhang [1 2 3 4 5]
1: Howard Hughes Medical Institute 2: Broad Institute of MIT and Harvard 3: McGovern Institute for Brain Research 4: Department of Brain and Cognitive Sciences, MIT 5: Department of Biological Engineering, MIT

Many powerful molecular biology tools have their origin in nature, and, often, microbial life. From restriction enzymes to CRISPR-Cas9, microbes utilize a diverse array of systems to get ahead evolutionarily. We are interested in exploring this natural diversity through bioinformatics, biochemical, and molecular work to better understand the fundamental ways in which living organisms sense and respond to their environment and ultimately to harness these systems to improve human health. Building on our demonstration that Cas9 can be repurposed for precision genome editing in mammalian cells, we began looking for novel CRISPR-Cas systems that may have other useful properties. This led to the discovery of several new CRISPR systems, including the CRISPR-Cas13 family that target RNA, rather than DNA. We developed a toolbox for RNA modulation based on Cas13, including methods for precision base editing. We are expanding our biodiscovery efforts to search for new microbial proteins that may be adapted for applications beyond genome and transcriptome modulation, capitalizing on the growing volume of microbial genomic sequences and building on our bioengineering expertise. We are particularly interested in identifying new therapeutic modalities and vehicles for delivering cellular and molecular cargo. We hope that this combination of tools and delivery modes will accelerate basic research into human disease and open up new therapeutic possibilities.

INV05

From TCRs to engineered T cell products

E D'Ippolito [1]
1: Technische Universität München

T-cell engineering with antigen-specific T-cell receptors (TCRs) allows the generation of increasingly specific and reliable T-cell products for cancer and viral infections. However, the path for identifying adequately functional TCRs and bring them into clinical applications is still tedious. We therefore developed platforms to support a rapid selection of candidate TCRs for therapy, and to ensure precise genome engineering for generating near-to-physiological TCR-engineered T cells.

The highly diverse antigen-unexperienced TCR repertoire of healthy donors represents a suitable source for identifying tumor-specific TCRs. Rare antigen-specific naïve T cells are enriched from large-size T-cell apheresis and single-cell sorted according to pMHC class I multimer staining. During cell sorting, our developed flow cytometry-based functional screening estimates the structural avidity of each individual pMHC multimer-reactive TCR, which correlates with functionality. Altogether, epitope-specific TCRs can be isolated and concurrently ranked according to predicted avidity/functionality. TCRs can also be efficiently isolated from antigen-experienced donors. Considering the higher frequencies of these memory repertoires, we developed a high-throughput, single-cell sequencing-based approach capable of discriminating highly functional and bystander TCRs according to transcriptional shifts in T-cell activation genes induced by recent peptide stimulation before cell sorting (Wagner et al., Cell Reports 2022; Mateyka et al., Vaccines 2022).

For TCR re-expression, we developed a non-viral CRISPR-Cas9-mediated engineering method, called orthotopic TCR replacement (OTR) (Schober et al., Nat Biomed Eng 2019). This approach allows us to integrate a transgenic TCR of interest in the endogenous TCR alpha locus. The simultaneous knockout of the TCR beta locus ensures the complete replacement of the endogenous TCR. Compared to other TCR-engineered T cell products generated with conventional viral transduction, the OTR technology ensures a physiological, well-defined and reproducible expression of the transgenic TCR. This ultimately results in a more predictable *in vivo* behavior of the T-cell product.

INV07

Deliver to the liver: towards one-and-done gene therapies for defects of hepatic metabolism

A Cantore [1 2]
1: San Raffaele Telethon Institute for Gene Therapy (SR-Tiget) 2: Vita-Salute San Raffaele University

The liver is an attractive target organ for *in vivo* gene therapy. Importantly, adeno-associated viral (AAV) vectors have recently obtained marketing authorization for adult patients affected by hemophilia. However, their use in pediatric patients is challenged by their non-integrating nature and consequent dilution of the episomal genome upon cell proliferation during liver growth. Lentiviral vectors (LV) are a promising alternative since they integrate into the target cells

genome. We achieved targeted transgene expression into hepatocytes, and stable gene transfer in adult mice, dogs, and non-human primates by LV intravenous (i.v.) administration. Here, I will first discuss the impact of spatio-temporal dynamics of hepatocytes during post-natal growth on transduction efficiency and transgene maintenance over time. We observed that LV i.v. administration to young mice leads to a 4-fold higher LV-positive liver area compared to adults. We also observed that only a fraction of hepatocytes proliferates during post-natal growth to generate the vast majority of the adult liver tissue. We thus performed spatial transcriptomics analysis of livers of young and adult mice to analyze changes in transcriptome profile that could affect hepatocyte permissiveness to LV transduction. In young mice, hepatocytes showed a peri-portal transcriptional profile, regardless of their localization in the lobule, lacking a fully established metabolic zonation. Interestingly we identified the proteasome pathway as a possible cell-intrinsic restriction factor, since it was highly expressed in adult peri-central hepatocytes compared to young livers. We administered a proteasome inhibitor to adult mice before LV and achieved 4-fold higher liver transduction compared to LV-only treated mice, indicating that proteasome plays an important role in determining the efficiency of LV hepatocyte transduction. I will then discuss possible applications of LV-mediated liver directed gene therapy to metabolic diseases mostly affecting young patients. One of those is familial hypercholesterolemia, FH, caused by mutations in low density lipoprotein receptor, LDLR, resulting in accumulation of circulating LDL and atherosclerosis. I.v. administration of LV expressing LDLR to juvenile FH mice achieved complete, long-term stable LDL normalization and prevention of atherosclerosis, even after challenge with high-fat diet. Another possible application is methylmalonic acidemia, a severe disease caused by deficiency of the mitochondrial enzyme methylmalonyl-coA mutase (MUT). The resulting build-up of toxic methylmalonic acid (MMA) leads to multisystemic life-threatening complications including chronic renal disease and neurological disabilities. Liver-directed LV gene therapy allowed rapid, substantial, and long-lasting decrease in circulating MMA, complete rescue of liver histopathology coupled to significant MMA reduction also in the brain and kidney, with correction of the mitochondrial alterations, in a mouse model of the disease. Overall, our data provide evidence for the efficacy and safety of liver gene therapy by LV for FH and methylmalonic acidemia in mouse models and will inform further development towards application to pediatric patients.

INV08

Understanding intracellular innate immunity and how to apply it to infection, cancer and gene therapies

G J Towers [1]
1: University College London

Gene therapy promises to transform human medicine. Hitherto untreatable diseases can now be effectively treated or even cured with modern gene therapy technology. Gene therapy depends on effective gene delivery and viruses make fantastic tools for this because they have evolved to deliver novel gene sequences to human cells for permanent or transient expression. Unfortunately, humans have evolved over millions of years to defend themselves from viral infection. Individual cells can effectively suppress infection using an intracellular innate immune system which depends on sensors called pattern recognition receptors (PRR) that detect incoming virus by sensing pathogen associated molecular patterns (PAMPs). Sensing activates kinase pathways and transcription factors which induce antiviral gene expression, particularly interferons, which are secreted to activate antiviral interferon stimulated gene (ISG) expression in nearby uninfected cells. Viral nucleic acids make particularly good PAMPs detected by DNA sensor cGAS or RIG-like RNA receptors. ISG are also expressed in uninduced cells that have not

detected infection. In fact, human stem cells tend to express high levels of specific ISGs and tend to be poorly responsive to IFN suggesting that stem cells represent a special case and are particularly good at defending themselves from infection. Typically, gene transduction depends on high dose vector to overcome target cell innate immunity. This works effectively, although it raises vector costs, and may induce unwanted inflammatory gene expression in transduced cells.

Our work aims to understand how intracellular innate immunity works and how viruses evade or antagonise its activities to support infection. A key goal has been to work out how to enhance vector infectivity through understanding and mitigating intracellular innate immune mechanisms. For example, we have developed transduction enhancers that inhibit the key stem cell-expressed antiviral IFITM proteins thereby facilitating use of lower vector doses for efficient lentivector transduction of human stem cells. We are also considering how innate immune activation in tumours is associated with outcome, particularly after immunotherapy, which seems to work best when innate immunity is activated. Here, a key goal is to understand the role, and mechanisms of regulation, of virus-like nucleic acids PAMPs expressed from the nucleus in activating innate immunity in cancer. We have also developed anti-HIV inhibitors that break open the HIV capsid to expose the nucleic acid contents to sensors thereby killing infection and simultaneously activating innate immune responses. We propose that understanding innate immune mechanisms can improve gene therapy further and will suggest novel therapeutics that leverage this highly evolved and effective defence system.

INV09

Intravitreal gene therapy for inherited retinopathies

S Michalakis [1]
1: LMU Hospital, LMU Munich

Inherited retinopathies are a group of diseases caused by pathogenic variants in one of more than 250 known disease genes. Depending on the gene affected, they can have different manifestations, ranging from mild visual impairment to complete blindness. So far only one authorized gene therapy exists for the treatment of a specific retinopathy caused by mutations in the *RPE65* gene. This gene therapy is based on recombinant adeno-associated virus (AAV) vectors and is administered locally under the retina by an invasive procedure known as a "subretinal injection" that involves retinal detachment. This is necessary because conventional AAV vectors must be applied directly to the cell surface to efficiently infect and transduce the target cells, in this case the retinal pigment epithelium (RPE). Retinopathies caused by mutations in genes expressed in rod and cone photoreceptors would also require subretinal injection. However, subretinal injection carries the risk of collateral damage and only treats a small portion of the affected retina. In addition, the retina of some retinopathy patients is often more vulnerable and susceptible to collateral damage from subretinal injection. One possible way to address this problem is to use next-generation AAV vectors that can bypass retinal barriers and transduce retinal target cells when administered via less invasive routes of administration such as intravitreal injection. Here, I will discuss recently developed intravitreal AAV gene therapy approaches for the treatment of retinitis pigmentosa and other retinopathies, including aspects of preclinical efficacy and safety testing and future clinical application.

INV10

Gene therapy for focal pharmacoresistant epilepsy

D Kullmann [1]
1: University College, London

Epilepsy affects 1% of the population, and one third of affected individuals continue to experience seizures despite medication, thus representing a very large unmet need. Because seizures frequently arise in a defined brain region, pharmacoresistant epilepsy is potentially amenable to targeted gene therapy. Some rare syndromes have an identifiable genetic cause, but in the majority of cases there is no clear molecular defect that can be corrected. Instead, seizures arise from an impairment of the normal mechanisms that restrain runaway excitation in cortical circuits. Several candidate gene therapies have been developed to alter the excitation/inhibition balance. Recent advances include chemogenetic strategies to allow the therapeutic effect to be controlled pharmacologically and closed loop approaches that allow circuit excitability to be suppressed selectively by pathological over-activity. I shall discuss preclinical studies that identify some of these strategies as especially promising for clinical translation.

INV13

CMC challenges in the development of gene therapies and how to address them – regulatory aspects

J Reul [1]
1: Paul Ehrlich Institute

Gene therapies are a heterogeneous group of medicinal products including cell-based and vector-based strategies and are classified in the EU as advanced therapy medicinal products (ATMPs) for which specific regulatory provisions are in place. Several products were put on the market in the last decade and numerous innovative strategies addressing a wide variety of indications are under development. However, translating scientific discoveries from bench to bedside involves a number of challenges. In respect to quality, manufacturing and control of gene therapies are tremendously complex. Due to their characteristics the finished products may often have high degree of variability and testing poses specific challenges, for example, due to sampling issues in case of autologous products, limited batch sizes, or short shelf life. For some gene therapies, such as genetically modified cells, the starting materials which may be viral vectors, mRNA or plasmids are complex in nature and require costly manufacturing processes and control strategies. In this presentation, CMC challenges on the way from bench to bedside and common issues in manufacturing and control of gene therapies in early clinical development will be discussed from a regulatory perspective. Strategies to overcome these challenges will be outlined.

INV17

Maximizing the exploitation of a massive metagenomic data for the advancement genome editing tools

A Cereseto [1]
1: University of Trento

CRISPR technologies initiated a new era for the advancement of genome editing. The new paradigm brought by the CRISPR technology is the concept of RNA guided nucleases as a mean to ease programmability of genome editing. Yet, various hurdles are limiting the desirable broader use of the genome editing applications. Challenges are imposed by various properties of CRISPR tools including poor compatibility with delivery systems, target sequence constraints and unpredictable efficiency and precision throughout the genome. We recently focused on the development of the technologies by scouting new systems existing in nature. To this aim we used a massive metagenomic database to identify new CRISPR systems and RNA guided nucleases with more favourable features for genome editing purposes including low molecular size. Since most novel systems derived from prokaryotes are non-functional in eukaryotic cells, we generated a directed evolution platform using eukaryotic cells, EPICA, allowing the generation of Cas variants with enhanced activity. Our discovery-enhancement pipeline will set the stage to unlock a large variety of nuclease tools matching the complexity of genome editing applications in eukaryotes.

INV19

AAV integration in preclinical models of gene therapy

D Cesana [1]
1: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET)

Adeno Associated Viral vectors (AAVs) have been successfully exploited in gene therapy (GT) applications for the treatment of several genetic disorders. Despite they are considered episomal vectors, it has been shown that fragmented or full-length AAV DNA can integrate within the genome of host cells leading to hepatocellular carcinoma and clonal expansion events in some preclinical models. Hence, a deeper assessment of AAV integration events is required.
We recently developed a sonication-based PCR approach and a novel bioinformatics tool, referred as RAAVIoli, allowing the identification and characterization of AAV integration sites (IS). Thanks to this platform, we investigated the distribution of AAV IS in different preclinical models of GT and gene editing (GE).

AAV IS were identified in the liver of mice and Non-Human Primates systemically injected with therapeutic AAVs. Liver-derived AAV IS displayed the previously reported preference to integrate close to CpG islands and around the transcription start sites (TSS) of targeted genes. A similar integration pattern was observed when AAV IS were retrieved from blood-derived cell-free DNA by LiBIS-seq, thus revealing the feasibility of the method for longitudinal safety studies in in vivo GT applications.

In clinically relevant GE contexts based on human hematopoietic stem cells (HSPCs) engrafted long-term in immune-deficient mice, we observed that AAV Inverted Terminal Repeats (ITRs) have a strong tendency of being captured at artificially induced double-strand breaks. Hence, the

presence of transcription-competent ITR fragments as an inadvertent consequence of HDR protocols may raise safety concerns regarding the use of this vector in GE applications. However, AAV integration can also be beneficial. We recently showed that T cells of immune-deficient ZAP70 knock-out mice intra-thymically injected with an AAV expressing the therapeutic transgene were characterized by clusters of AAV vector integrations in T-cell receptor genes. The insertions mapped to DNA breaks created by the enzymatic activity of recombination activating genes (RAG) during V(D)J recombination. ITRs were mainly involved in those integration events, confirming their intrinsic and strong tendency to be captured at DNA damage sites by non-homologous end-joining mechanisms. These integrations allowed the long-term transgene expression in functional and proliferating T lymphocytes, providing therapeutic benefits. Similar integration results were also obtained in wild-type mice intra-thymically injected with a GFP-expressing AAV, indicating that this delivery route induces a site-specific integration of AAV genomes in T cell progenitors. As peripheral gene-corrected T cells can persist over long periods of time, this new “targeting” approach can open therapeutic avenues for achieving long-term AAV-mediated gene transfer in dividing T cells.

Overall, these findings indicate that AAV integration studies offer significant insights for improving the safety and efficacy of GT treatments.

INV21

Advances and pitfalls in AAV therapy targeting inherited cholestasis

N D Weber [1] L Odriozola [2] C Bouquet [1] C Gázquez [2] A Douar [1] J P Combal [1] T K Kishimoto [3] C Smerdou [2] G González-Aseguinolaza [1 2]
1: Vivet Therapeutics 2: Centro de Investigacion Medica Aplicada (CIMA) 3: Selecta Biosciences

Inherited cholestasis is a group of rare genetic disorders characterized by impaired bile flow, leading to severe liver dysfunction and fibrosis. Among these disorders, Progressive Familial Intrahepatic Cholestasis Type 3 (PFIC3) is particularly devastating, with limited treatment options and a high mortality rate. It arises from loss-of-function mutations in the *ABCB4* gene that encodes MDR3, a phosphatidylcholine transporter protein involved in bile composition homeostasis. We have made several strong advances towards developing and testing an AAV-based gene therapy designed around *ABCB4* gene supplementation for PFIC3 treatment.

In both adult and infant *Abcb4*$^{-/-}$ mice, all disease parameters have been fully normalized to wild-type levels, including serum biomarkers of liver damage, hepatosplenomegaly, biliary phosphatidylcholine levels and liver fibrosis, following treatment with an AAV8 vector harbouring human *ABCB4* cDNA. These outcomes were achieved both in mice with moderate liver fibrosis and ones with more advanced disease including severe fibrosis/cirrhosis at time of treatment, and resulted in a long-term cure. Additionally, successful short-term repeat administration has been achieved in infant mice via coadministration with the immunotolerogenic nanoparticle ImmTOR together with the AAV. The success of this strategy hinged on preventing the generation of AAV capsid-specific neutralizing antibodies, which severely inhibit transduction of any subsequent AAV treatment and is a major hurdle for AAV therapy for patients previously exposed to AAV. Furthermore, this therapeutic strategy resulted in a prevention of the development of hepatocellular carcinoma in these mice, which are highly prone to its appearance.

Despite these advances, multiple obstacles remain. Thus far, high vector doses have been required, which may be a result of the mouse model, liver fibrosis at time of treatment, or both,

but is of paramount importance. High vector dose requirements cause issues in manufacturing costs and more importantly, have been associated with severe adverse events in the clinic, including death. Immunological responses, immunological memory against AAV and a loss of effect due to the dilution of AAV episomes, particularly in liver diseases with high hepatocyte regeneration such as PFIC3, are other issues confronting this therapy. Not to be overlooked are the facts that patients for this treatment will predominantly be children, and they will have fibrotic livers at the time of treatment, both issues that strongly influence dosing, safety and AAV transduction efficiency. However, with continued efforts, AAV gene therapy has immense potential to revolutionize the management of this life-threatening condition.

INV22

Systemic and local immune responses to ocular gene therapy

D Dalkara [1]
1: Institut de la Vision, Paris

For over a decade, researchers have successfully used gene therapy either in the form of gene replacement or gene editing in clinical trials for inherited retinal diseases. However, in some cases, patients experience inflammation, necessitating corticoids as an additional treatment. This raised questions about the immune effects of gene therapy administration in this immune privileged organ. To investigate immune reactions to ocular gene therapy, we conducted a battery of experiments in mice and non-human primates. In non-human primates, we investigated the effect of AAV dose and administration route in relation to circulating antibody titers. In rodents, we carried two sets of experiments involving AAV mediated gene delivery or delivery of CRISPR therapeutics. We observed that subretinal AAV injections led to proinflammatory T-cell responses against the transgene product, particularly in cases where high doses of AAV were used. Interestingly, co-injecting immunodominant peptides from the transgene product alongside the AAV attenuated the immune response at all tested AAV doses. Our findings indicate that proinflammatory T-cell responses to the transgene product after subretinal AAV injections can be modulated by the subretinal-associated immune inhibition (SRAII) mechanism. Additional mechanisms are likely in play when CRISPR therapeutics are administered in protein form and may benefit from immunomodulation strategies.

INV25

Base editing and prime editing: correcting mutations that cause genetic disease in cells, animals, and patients

D Liu [1]
1: Broad Institute, Harvard University, and HHMI - Cambridge, MA

In this lecture I describe the development and therapeutic application of two precision gene editing technologies that install or correct targeted mutations without requiring double-strand DNA breaks, thereby minimizing undesired consequences of chromosomal cleavage. We developed base editors, proteins that directly perform chemistry on individual DNA bases in living

cells to install or correct mutations at targeted positions in genomic DNA. We recently engineered CRISPR-free, all-protein base editors that enabled the first purposeful changes in the sequence of mitochondrial DNA in living cells. By integrating base editors with *ex vivo* and *in vivo* delivery strategies that deliver therapeutic proteins, we rescued animal models of human genetic diseases including sickle-cell disease, progeria, and spinal muscular atrophy (SMA). Single-AAV base editing systems enhance the safety and practicality of *in vivo* base editing. Our development of engineered virus-like particles (eVLPs) provide additional *in vivo* delivery methods for gene editing proteins that minimize off-target editing and the risk of oncogenic DNA integration. Base editors are in at least six clinical trials to treat diseases including familial hypercholesterolemia, sickle-cell disease, beta-thalassemia, and T-cell leukemia, resulting in the first report of clinical benefit from a base edited therapeutic. I will also describe prime editors, engineered proteins that directly write new genetic information into a specified DNA site, replacing the original sequence, without requiring double-strand DNA breaks or donor DNA templates. Prime editing can mediate any base substitutions, deletions, and/or insertions of up to ~200 base pairs in living cells *in vitro* and *in vivo*, and has been applied to directly install or correct pathogenic alleles that previously could not be corrected in therapeutically relevant cells. We illuminated the cellular determinants of prime editing outcomes, and used the resulting insights to develop new prime editing systems with substantially higher editing efficiencies and product purities. Most recently, we used phage-assisted continuous evolution (PACE) to evolve a suite of sixth-generation prime editors (PE6a-6g), which each evolved to specialize in different types of prime edits. The combination of prime editing and site-specific recombinases enable programmable gene-sized (>5 kb) integration and inversion at loci of our choosing in human cells. Prime editing has recently been used to rescue animal models of genetic diseases including sickle-cell disease, metabolic liver diseases, and genetic blindness. Base editing and prime editing enable precise target gene correction, in addition to target gene disruption, in a wide range of organisms with broad implications for the life sciences and therapeutics

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INV27

mRNA Galsomes - making mRNA vaccines more effective, broadly applicable and well-controlled

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1: Ghent University 2: VIB-UGent

mRNA vaccines have gained global recognition for being generally safe and highly effective although questions remain on the mode of action and inflammatory nature of this novel vaccine platform (Verbeke R. et al. JCR 2021). Within our research group, we developed the mRNA Galsome platform where N1-methyl-pseudouridine (m1Ψ) modified mRNA vaccines are co-formulated with the potent broad-spectrum adjuvant alpha-Galactosylceramide (α-GalCer). The unique value of α-GalCer is that it empowers conventional T cell responses while simultaneously broadening the immune response by activating NKT- and NK cells (Verbeke R. et al. ACS Nano 2019) which is specifically of interest for the development of vaccines against intracellular bacterial infections and therapeutic cancer vaccines.

For the latter, we are setting-up a phase I clinical trial in lung cancer patients in close collaboration with Ghent University Hospital. Besides establishing a GMP-compliant production system, the clinical translation process comprised the fine-tuning of the LNP formulation, mRNA construct and administration route where we investigated the impact of these parameters on the reactogenicity and immunogenicity with an emphasis on the induction of robust T cell responses in mice. The highest antigen-specific T cell responses were obtained after IM injections of mRNA-

LNPs containing αGC with a clear dose-sparing effect exerted by the adjuvant. We also observed clear differences in reactogenicity using different LNP formulations which was also further confirmed in an *ex vivo* human PBMC model.

Within the context of the EU consortium “Baxerna2.0” we are currently also exploring the development of mRNA Galsomes for prophylactic vaccination against intracellular bacterial infections like Mycobacterium Tuberculosis. As a first proof of concept, we illustrated a clear increase in protection against a Listeria Monocytogenes infection after vaccination with mRNA Galsomes encoding a newly discovered Listeria antigen (Mayer et al., 2022).

Taken together, we believe that the mRNA-Galsome platform consisting of α-GalCer-adjuvanted mRNA-LNPs holds great potential for those applications where a multifaceted immune response is warranted.

INV28

Building better: engineered B Cells as an evolving immunotherapy for fighting escape-prone diseases

A D Nahmad [1]
1: Tabby Therapeutics

HIV viremia may be controlled by chronic antiretroviral therapy. However, medication compliance remains an issue particularly as individuals age and face polypharmacy induced side effects.

To address these challenges, we explored an innovative single-shot alternative: transplantation of ex-vivo engineered B cells (EBCs). Our targeting scheme delivers an antibody coding cassette to the native antibody heavy chain locus. Upon immunization, adoptively transferred EBCs home to germinal centres, where they dominate over the endogenous response. Our engineering approach allows class switch recombination to occur with the transgenic antibody, potentially potentiating Fc receptor mediated activity and permitting tissue wide immune surveillance. Furthermore, the antibody coding gene undergoes somatic hypermutation, enabling affinity maturation and clonal expansion of clones that are more competitive, potentially limiting viral escape. In B cells, the delivered antibody is expressed as a B cell receptor, enabling antigen induced activation. In antibody secreting cells, the cassette undergoes alternative splicing, permitting secretion. Indeed, EBCs can differentiate into plasma cells to secrete high and neutralizing antibody titers in mice following immunizations and this effect is further amplified upon boost immunizations, exemplifying memory retention.

We also highlight that B cells can be engineered in-vivo, using two adeno-associated viral vectors. We observed successful editing of B cells leading to memory retention and antibody secretion at neutralizing titers. Minimal nuclease off-target cleavage was detected and on-target cleavage in undesired tissues is reduced by expressing Cas9 from a B cell-specific promoter.

Beyond viral infections, B cell engineering could be exploited for the treatment of cancer. Recent breakthroughs revealed that B cells are linked to favorable prognosis and promote response to immunotherapy. We demonstrate that B cells can be engineered to express anti-oncoantigen antibodies and reach high efficiencies in mouse and human B cells. EBCs internalize and process the antigen for MHC presentation, enabling oncoantigen specific T cell activation in-vitro. In-vivo, EBCs migrate to TDLNs and form germinal centres. Slower tumor growth and

improved survival was detected in tumor bearing, EBC recipient mice in comparison to non-engineered B cell recipient mice.

In conclusion, our findings highlight the potential of B cell engineering as a versatile therapeutic platform for combating infectious diseases and for enhancing anti-tumour responses in cancer treatment. Engineered B cells present a promising approach to address escape-prone diseases and should be considered as evolving therapeutics in the pursuit of effective immunotherapies. Moreover, in-vivo B cell engineering offers a scalable solution to reduce production hurdles, opening new avenues for future clinical applications.

INV32

Accessibility of haemophilia Gene Therapy

B O'Mahony [1]
1: Irish Haemophilia Society

Access to gene therapy for people with haemophilia will have to be part of a shared decision making process between the individual patient and doctor, ideally with the relevant patient organisation providing supportive information and education. There are now several generations of therapies available for haemophilia and the decision to be treated with gene therapy needs to be fully cognisant of the known facts and the uncertainties in areas including factor expression, durability, predictability and long term safety. Accessibility will also depend on affordability which may well require new payment models to deal with the uncertainty and pay on an outcome or annual payment model basis for a one-off therapy.

INV34

Ensuring access to life-saving gene therapy for an ultra-rare disease: a not-for-profit model

S Benvenuti [1] F Miotto [1] S Russell [1 2]
1: Fondazione Telethon 2: PrimeRA Pharma Partners

Fondazione Telethon is a charity funded 30 years ago by a patient organization (Unione Italiana Lotta alla Distrofia Muscolare – UILDM) with the mission to advance biomedical research towards the cure of rare genetic diseases. One of its most significant success stories was the EMA approval, back in 2016, of Strimvelis®, the first ex-vivo gene therapy approved worldwide. This therapy intends to treat children affected by severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) who lack a matching bone marrow donor. ADA-SCID is an inherited disorder caused by mutations in the ADA gene and results in a seriously compromised immune system. People affected by ADA-SCID almost lack all immune protection from bacteria, viruses and fungi becoming prone to repeated and persistent infections that can be very serious or life-threatening. Most patients are diagnosed in the first 6 months of life and, without treatment, they usually die before 2 years of age.

Strimvelis® was developed by the San Raffaele Telethon Institute for Gene Therapy in Milan (SR-TIGET), Italy who then partnered with GlaxoSmithKline to complete the CMC activities of scaleup and validation of vector and transduced cells manufactured at the Italian contract manufacturing organisation, MolMed (now AGC biologics). In 2018 the marketing authorization was transferred to Orchard Therapeutics Ltd (OTL).

At the end of March 2022, OTL announced that it will discontinue the programme. As no other alternative therapies are available for those patients, Fondazione Telethon decided to step-in and started a negotiation with OTL to ensure Strimvelis® will remain available to the patients.

In July 2023 the transfer of the marketing authorization from OTL to Fondazione Telethon was approved by the European Commission making Fondazione Telethon the first not-for-profit organization to become marketing authorization holder of an ATMP in Europe.

Fondazione Telethon has faced and is still facing big challenges in this process:

- Financial constraints as the commercialization of Strimvelis at the current price is economically challenging even for an organization that does not look for a profit nor is looking to recover the investments made during the development phase
- Organization and human resources – to become and act as a marketing authorization holder Fondazione Telethon had to recruit staff from the pharmaceutical sector, revise its organizational chart to accommodate new functions and reorganize its internal structure and processes.
- Being Strimvelis the only product distributed by Fondazione Telethon, the fix costs to maintain the necessary (minimum) structure have an high impact on the cost per patient.
- The current regulatory environment does not foresee any incentive in the post-marketing phase for a not-for-profit organization that does not meet the criteria to be considered as a “small and medium enterprise – SME”.

Fondazione Telethon decision could pave the way for a different commercialization model of ATMPs for ultra-rare diseases but such a model should apply only where there are no other alternatives. This model is not an alternative to the standard pharmaceutical development, it could be complementary where the current for-profit model cannot find a commercial sustainability for those products.

INV36

Preclinical and early clinical evaluation of infectivity- and potency-enhanced oncolytic adenoviruses ORCA-010 and ORCA-020

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1: Dept. Medical Oncology, Amsterdam UMC, Cancer Center Amsterdam, Vrije Universiteit Amsterdam, Amsterdam, The Netherlands 2: ORCA Therapeutics BV, ’s-Hertogenbosch, The Netherlands

Oncolytic adenoviruses are promising immunotherapeutic agents for the treatment of cancer. Current generation viruses have shown promising clinical successes, but also room for improvement. We aim to further improve treatment efficacy by strengthening the lytic potency and immune stimulatory capacity of oncolytic adenoviruses. The presentation will include: (1) Results of an ongoing phase 1/2a study with ORCA-010 (Ad5-D24.RGD.T1) in treatment-naïve

patients with localized prostate cancer. ORCA-010 carries mutations providing cancer-selective replication, increased cell infection and accelerated cell lysis to promote virus release. The single dose escalation part 1 of the clinical trial is completed; and at the time of the presentation inclusion of repeat dosing part 2a should be completed. Safety, biological activity (virus replication and immune cell infiltration) and efficacy results (PSA, MRI) will be presented. One year after treatment, tumor biopsies revealed increased immune cell infiltrates, suggesting durability of immune responses. (2) Preclinical evaluation of ORCA-020 (Ad5-D24.RGD.T1-CA.GSK3b) in human melanoma-immune cell co-cultures *in vitro*. ORCA-020 was designed because melanoma-intrinsic active β-catenin signaling can cause T-cell exclusion from the tumor microenvironment contributing to resistance to immunotherapy. ORCA-020 expresses constitutively active GSK3b from the ORCA-010 backbone to promote b-catenin degradation in infected cancer cells. The oncolytic replication properties of ORCA-010 and ORCA-020 were similar in human cancer cell lines, but ORCA-020 bolstered immune cell activation. CA.GSK3b expression counteracted melanoma cell-induced suppression of dendritic cell development and activation. Together, these findings provide guidance for further development of oncolytic immunotherapy procedures.

INV39

A novel approach for the treatment of inborn errors of metabolism that benefit from HSCT: development of FBX 101 for the treatment of Krabbe disease

M Escolar [1]
1: Forge Biologics

For more than 30 years, many metabolic conditions have benefited from hematopoietic stem cell transplantation (HSCT). However, the treatment may not be fully effective as is the case of some lysosomal storage and peroxisomal disorders. A myeloablative chemotherapy followed by several weeks of immune suppression are necessary to open space in the marrow for the new hematopoietic graft and to prevent graft versus host disease. Taking advantage of the inability of the host to build an immune response, we have studied the effects of administering intravenous AAV during this period and evaluate if immune responses against the transgene and the capsid could be prevented. Immune responses have been historically one of the biggest challenges of AAV therapy, preventing redosing and treatment of subjects previously exposed to AAV. An increased antibody response can neutralize the effectiveness of AAV therapy and in a few cases, result in multiple organ failure and death. We will discuss our experience in a phase I/II trial using FBX101, an AAVrh10.hGALC administered intravenously after transplantation for infantile and late infantile Krabbe disease. Krabbe disease is a lysosomal storage disorder and severe leukodystrophy characterized by rapid demyelination that leads to death at an average of 2 years (in the more common infantile onset form) and by 6 years in the late infantile form. For the last 18 years, the standard of care has been hematopoietic stem cell transplantation (HSCT) for those patients with the infantile and late infantile form diagnosed while asymptomatic and those with the late infantile form who are mildly symptomatic. HSCT results in significant improvements in the myelination of the brain but there is continued disease progression of the peripheral nerves leading to death by teen years and early adulthood. We will present the safety and exploratory efficacy of 5 patients treated systemically with FBX101 administered as early as 21 days and as late as 4 years after HSCT. We will discuss the broader implications and potential ability to re-dose patients treated during myelo/immune ablation and the benefits of using unrelated umbilical cord blood as donor from the immunological perspective. Lastly, we will discuss the potential of this approach for other diseases that already benefit from HSCT.

INV41

Improving the efficiency and precision of gene editing in hematopoietic stem cells

D Bauer [1]
1: Harvard Medical School

Gene editing of hematopoietic stem cells promises to definitively treat a variety of inherited and acquired blood disorders. To maximize its potential, the gene edits must be efficient and precise. Here I will discuss several considerations to optimize gene editing outcomes in hematopoietic stem cells for clinical purposes that cannot be determined by studies in cell lines alone. First, I will consider how genetic variation can alter the propensity for variant-specific off-target effects. I will present an example of a common genetic variant associated off-target that may produce pericentric inversions in therapeutically gene edited hematopoietic stem and progenitor cells. Also, I will discuss a versatile publicly available tool to nominate such off-target potential. Second, I will review how the quiescent nature of hematopoietic stem cells, while limiting DNA repair pathways available for therapeutic gene editing, may in fact protect the cells from genotoxicity including micronucleus formation and long deletions. Finally, I will examine how the quiescent state of hematopoietic stem cells may constrain the efficiency of therapeutic prime editing.

INV42

Genetically modified epidermal stem cell: a treatment for Epidermolysis Bullosa

L de Rosa [1]
1: University of Modena and Reggio Emilia

Epidermolysis Bullosa (EB) is a genetic skin disease that can be very severe and even lethal in some cases. Although there is no definitive cure for EB, recent progress in molecular and cellular biology is leading to the development of advanced therapeutic strategies. Here we present one of the more challenging and ambitious therapies, which involves regenerating a fully functional epidermis by targeting epidermal stem cells. This therapy offers a therapeutic option for JEB patients with LAMB3 mutations and it is paving the way for a combined cell and gene therapy platform that can tackle other forms of EB and other genodermatoses.

INV43

Rapid and highly efficient transient redosable gene therapy

S Krishnan [1]
1: Krystal Biotech, Inc., 2100 Wharton St, Suite 701, Pittsburgh, PA

Herpes simplex virus type 1 (HSV-1) is a DNA virus with many favorable properties both as a delivery vector for therapeutic genes and as a backbone. HSV-1 is an infectious virus, so they are efficient vehicles for the delivery of exogenous genetic materials to patient cells. The inherent cytotoxicity of this virus, if harnessed and made to be selective by genetic manipulations, makes this virus a good candidate for treating genetic diseases. Furthermore, its large genome size, ability to infect cells with a high degree of efficiency, and the presence of an inherent replication controlling mechanism, the thymidine kinase gene, add to its potential capabilities. Cytotoxicity of the virus is abolished by deleting specific viral gene products and its large genome size affords the virus a large payload capacity to carry genetic materials to patient cells. The virus grows well in tissue culture and high titer stocks can be obtained to treat the broader population. The virus is episomal and does not integrate into the host genome. These features enable the virus to deliver therapeutic genes while avoiding the potential for insertional mutagenesis.

The genetic medicines that Krystal Biotech is currently developing are designed to introduce therapeutic genes into a patient's cells. The genes are introduced into these cells use an engineered HSV-1 virus to render the virus harmless to the patient by modifying it to be non-replicating and non-integrating to the cell's native genetic materials. Once the modified viral vector has delivered the genes into the skin cells, the cell machinery then translates the delivered genes to express the encoded functional protein and providing and treating the underlying disease.

In summary, he use of our proprietary, modified HSV-1 as a gene therapy platform has a number of distinct advantages over other viral gene therapy vectors - 1) multiple routes of administration including topically 2) efficiency of therapeutic gene transfer as it transduces both dividing and non-dividing cells 3) transient transgene expression allowing for repeat administration 4) accommodate large or multiple copies of genes including large high payload capacity and 5) does not insert itself into, or otherwise disrupt the human genome.

INV44

Delivery and expression of mini- and micro-dystrophins for gene therapy of DMD

J S Chamberlain [1 2] S D Hauschka [1 2] H Tasfaout [1 2]
1: University of Washington 2: Wellstone Muscular Dystrophy Specialized Research Center

DMD is caused by defective expression of dystrophin, which is encoded on the largest known gene (2.2 MB). Our previous studies led to the identification of AAV vectors as a vehicle that can systemically delivery genes to muscles bodywide. However, AAV can only carry ~5 kb of DNA, a limitation we partially overcame via structure/function studies of dystrophin to develop "micro-

dystrophins" (proteins ~1/3rd the size of dystrophin). Delivery of micro-dystrophins (µDys) under control of muscle-restricted enhancer/promoter elements derived from the MCK gene can halt and reverse dystrophic pathology in animal models for DMD. Our first generation AAV-MHCK7–H2µDys vector was adapted by Sarepta and led to the recent approval by the FDA for marketing. Unfortunately, early generation µDys vectors have not proven to be as robust in the clinic as in animal models due to AAV dose limitations and the small size of µDys proteins. Consequently, we have designed and tested newer generation micro-dystrophins that incorporate alternate domains to increase function, including in cardiac muscle, where early generation vectors are less robust. In addition to improved micro-dystrophins we have also tested numerous regulatory cassettes based on the MCK enhancer plus promoter to increase expression in striated muscles. We have also been developing novel vectors able to deliver dystrophins more than twice as large as µDys, including the full-length 427 kDa muscle isoform of dystrophin. These larger sizes enable inclusion of more functional domains, such as those needed for satellite cell activation. This new system relies on multi-vector delivery of partial dystrophin sequences carrying split-inteins, which leads to covalent joining of the fragments into larger proteins inside muscles. Using newer myotropic AAVs we achieve higher levels of mini- and full-length dystrophins after systemic delivery to dystrophic models using total vector doses 1/10th those being used in the clinic. This split intein system leads to better and faster functional correction of dystrophy compared with µDys proteins.

INV45

In vivo hematopoietic stem cell gene therapy

H P Kiem [1]
1: Fred Hutchinson Cancer Research Center, Seattle

Despite tremendous progress in the hematopoietic stem cell (HSC) gene therapy and gene editing fields, there are a number of significant limitations to the availability and accessibility of these therapies. In my presentation, I will discuss our data for better targeting HSCs for ex vivo and in vivo gene therapy applications. I will discuss different delivery platforms for in vivo gene therapy and how these approaches could make gene therapy much more portable and accessible for many diseases including for patients with hemoglobinopathies. In vivo gene therapy would not require highly sophisticated cell manufacturing facilities and thus could be made more easily available for patients in low and middle-income settings.

INV46

Updates and immune profiling in AAV gene therapy trials for patients with Tay-Sachs and Sandhoff diseases

T R Flotte [1] A Keeler [1] M Blackwood [1] R Artinian [1] H G Edwards [1] T Taghian [1] M S Esteves [1] O Cataltepe [1] F Eichler [2]
1: UMass Chan Medical School 2: Massachusetts General Hospital

The GM2 gangliosidoses, Tay-Sachs and Sandhoff diseases, are caused by deficiency of lysosomal

hexosaminidase A activity due to mutations in either the alpha subunit (encoded by HEXA) in Tay-Sachs or the beta subunit (encoded by HEXB) in Sandhoff. Severe deficiency states (≤ 0.1% activity) result in infantile forms of these diseases in which infants begin to show normal neurodevelopmental progress, but then begin to relentlessly regress around 5 to 6 months of age leading to global neurological deficits, seizures and ultimately death around 3 to 4 years old. Our group has developed rAAVrh8 vectors encoding HEXA and HEXB, which when injected simultaneously into the thalami bilaterally and into the CSF of deficient animals (Sandhoff mice and cats and Tay-Sachs sheep) can restore CNS Hexosaminidase enzyme activity, decrease GM2 levels in CSF, and rescue most or all of the phenotypic consequences of the disease. Following upon a small (N of 2) expanded access (EA) trial, we initiated a phase 1/2, single-dose, dose-escalation of combined biThalamic (BiT), intra-cisterna magna (CM), and intrathecal (IT) infusion of rAAVrh8-HexA/HexB at total doses ranging from 5.87E+12 vg/kg per patient to 4.08E+13vg/kg. Most importantly, the BiT injection volume (and vector dose) were doubled between each cohort (starting dose (SD) 180mcl, low dose (LD) 360mcl, mid dose (MD) 720 mcl, and high dose (HD) 1250 mcl) with the SD matching the dose used in the prior EA trial. Patients were immune suppressed with rituximab, sirolimus, and corticosteroids (10mg/kg solumedrol). Post-dose, patients continued with sirolimus for 6 months followed by a taper and prednisolone (1 to 2 mg/kg/day) for 3 months followed by a taper. To date, a total of 8 subjects have been enrolled in this trial. Interim results of CSF HexA enzyme activity and GM2 levels show a dose-related, partial biochemical correction of the disease. Positive clinical findings in treated infantile subjects indicate prolonged oral feeding without aspiration until age 3 to 3.5 years. The data on dose-response effects on the CSF biomarkers and clinical outcomes will support later development. Multiple aspects of the immune response were evaluated in these subjects, including cytokine responses, anti-capsid antibodies, anti-capsid and anti-transgene ELISPOT responses, and development of vector-specific Tregs. The timing of these responses and their treatment will be discussed in the context of broader application to other rAAV clinical trials. **Funded by NTSAD, CTSF, MFRF and BluGenes.**

INV48

From pivotal study to market approval: important findings for the successful use of gene therapy in haemophilia B

W Miesbach [1]
1: Goethe University Hospital in Frankfurt

Three decades after the first gene therapy approaches, the first treatment for severe to moderate haemophilia B was granted approval. This presentation will provide a comprehensive overview of phase 1-3 trials in haemophilia B, highlighting both similarities and differences among various approaches, as well as issues related to adverse events and durability. Additionally, the presentation will explore the array of immunosuppressive strategies implemented in clinical trials of haemophilia B gene therapy.

Two phase 3 trials, BENEGENE-2 (fidanacogene elaparvovec) and HOPE-B (etranacogene dezaparvovec), enrolled a total of 99 patients. The corresponding phase 1 studies had constant factor IX expression levels over a five-year period. While inclusion and exclusion criteria were largely similar in these studies, it is noteworthy that in HOPE-B, pre-existing anti-AAV (adeno-associated virus) antibodies were not considered an exclusion criterion.

Throughout the 24-month follow-up period, mean FIX expression remained stable at 25% for fidanacogene elaparvovec and 36.7% for etranacogene decaparvovec. In addition, both treatments showed a substantial and durable reduction in annualized bleeding rates (ABR) of 71% for fidanacogene elaparvovec and 80% for etranacogene decaparvovec, surpassing the efficacy of prior factor IX prophylaxis.

Of note, after treatment with fidanacogene elaparvovec and etranacogene decaparvovec, ALT (alanine aminotransferase) elevation occurred in 26.6% and 20.4% of cases, respectively. Encouragingly, no cases of FIX inhibitors, thrombosis, or gene therapy-associated malignancies were reported.

With the approval of the breakthrough gene therapy for haemophilia B, HEMGENIX®, in both the United States and the European Union, careful patient selection, vigilant post-treatment monitoring, and comprehensive data collection in national and international registries have become essential.

INV51

Design of lipid nanoparticles for enabling gene therapies

P Cullis [1]
1: University of British Columbia

Gene therapies employing genetic drugs such as small interfering RNA (siRNA) for gene silencing and messenger RNA (mRNA) for gene expression have the potential to cure most diseases. However, sophisticated delivery systems are required to enable the therapeutic use of nucleic acid polymers as they are quickly broken down in biological fluids, do not accumulate at sites of disease and cannot penetrate into target cells even if they arrive at target tissues. Lipid nanoparticle (LNP) technology is increasingly enabling the clinical potential of genetic drugs by packaging the nucleic acid in well-defined nanoparticles that protect the payload following systemic in vivo administration and facilitate intracellular delivery following uptake into target cells by endocytosis.

The first clinical validation of this approach was achieved by the approval of Onpattro by the US FDA in 2018 to treat the disease transthyretin-induced amyloidosis (hATTR. Onpattro consists of LNPs containing siRNA that silences production of transthyretin in the liver (in hepatocytes) following intravenous administration. In this talk I will describe the historical development of LNP systems leading to the development of Onpattro and how related LNP delivery technology is being employed to enable many mRNA-based gene therapy drugs. A notable example of the success of this approach is the development of Comirnaty, the Pfizer/BioNTech COVID-19 mRNA vaccine, which has played a leading role in alleviating the Covid-19 pandemic.

INV52

Development of broadly protective influenza vaccines using nucleoside-modified mRNA

N Pardi [1]
1: University of Pennsylvania

Influenza virus is one of the most important human pathogen. The influenza mortality is estimated to be approximately 650,000 per year worldwide, in addition, occasional global pandemics can infect up to 20-40% of the world's population. Licensed influenza virus vaccines require annual reformulation and readministration due to poor IgG longevity and lack of neutralization of related viruses. Development of a universal influenza virus vaccine with the potential to elicit long-lasting, broadly cross-reactive immune responses is necessary for reducing influenza virus prevalence. We have utilized lipid nanoparticle-encapsulated, nucleoside-modified mRNA vaccines to deliver a combination of influenza A group 1 or influenza B antigens to induce strong immune responses with substantial breadth and potency in a murine model. A single immunization with 50 ng of combined influenza A group 1 or combined influenza B vaccines induced protective immune responses against a broad panel of group 1 or influenza B viruses, respectively. These findings support the advancement of nucleoside-modified mRNA-lipid nanoparticle vaccines expressing multiple antigens as universal influenza virus vaccine candidates.

INV53

New targets and technologies for CAR T cells

M Hudecek [1]
1: University of Würzburg

Immunotherapy with gene-engineered immune effector cells has accomplished It's breakthrough in the treatment of malignant and non-malignant diseases. This talk will provide an update on target discovery and validation, synthetic immune receptor design and function, advanced virus-free gene-transfer and gene-editing technologies, as well as concepts for automated, scalable manufacturing - exemplified by specific use cases from the pipeline of CAR-modified immune cells at Universitätsklinikum Würzburg and several national and European network projects.

INV55

Not all roads lead to Rome

M Cabanes Creus [1]
1: Children's Medical Research Institute, Sydney

Seven years ago, the essential receptor for adeno-associated virus infection, KIAA0319L (now named AAVR) was discovered. Despite its significance, the function of this protein in AAV

transduction remains poorly understood. Independent studies have shown AAV2's physical interaction with AAVR. The absence of AAVR results in a block in AAV2's transduction pathway.

Our research using HuH-7 naïve and AAVR-KO HuH-7 cells suggests that while AAV2 vectors can enter cells lacking AAVR, this does not lead to RNA or protein expression. Using transmission electron microscopy, we found signs of nuclear envelope breakdown in naïve cells consistent with signatures of nuclear entry of other parvoviruses. This phenomenon was absent in AAVR-KO cells, indicating a block in the transduction pathway before nuclear entry.

While AAV2 in AAVR-KO cells mirrors the behavior of AAV2 virions missing the VP1 capsid protein, our data challenges the prevailing idea that endosomal acidification allows for VP1u-mediated escape. Instead, our findings support that the interaction of AAVR with the retromer complex may facilitate AAV's journey to the trans-Golgi. The absence of this interaction in AAVR-KO cells could explain the reported trapping of vectors in the endosomes, thus preventing functional AAV2 transduction.

Overall, the talk will provide an overview of the AAV trafficking pathway, integrating our new findings with trafficking data published in the literature.

INV56

Innate immunity to AAV vectors: the devil's in the details

G Ronzitti [1]
1: Genethon, Evry

The clinical reality of adeno-associated virus (AAV) vectors-mediated liver gene transfer is clearly supported by the recent approvals of Roctavian and Hemgenix, two gene therapy approaches for hemophilia A and B, respectively. However, clinical experience suggests that further optimization of the approach is needed to improve safety and long-term persistence of AAV gene transfer to the liver.

The liver sinusoidal endothelium, while providing a relatively free access to gene therapy vectors, also represents a lax barrier for the immune system cells that visit and populate the liver at high frequencies. Given its central role in the catabolism of food-derived proteins, the immune environment of the liver tends to be pro-tolerogenic. However, multiple conditions may unbalance the liver immune response toward immunogenicity.

Innate immunity is the first barrier of defense toward external aggressions. AAV vectors capsid and genome can be recognized by the innate immune system as foreign and activate anti-viral responses in particular when used at high doses. Here, I will discuss strategies to mitigate these responses including AAV capsid and genome engineering and immunosuppression. All these strategies may face liver conditions that may shift the balance toward immunogenicity, and I will focus on a model of liver fibrosis who seems to well recapitulate such conditions.

In the coming future, the use of such models, by considering the limitations imposed by a non-normal liver may allow to develop the next generation of gene transfer strategies with improved safety and long-term persistence.

INV57

Nanoblades allow high-level genome editing in murine and human organoids

V Tiroille [2] A Krug [2] E Bokobza [2] M Kahi [2] M Bulcaen [3] M M Ensinck [3] M H Geurts [4] D Hendriks [4] A Gutierrez-Guerrero [1] L Medaer [3] R Gijsbers [3] P E Mangeot [1] H Clevers [4] M S Carlon [3] F Bost [2] E Verhoeyen [1 2]
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Genome engineering has become more accessible thanks to the CRISPR/Cas9 gene editing system. However, using this technology in synthetic organs called 'organoids' is still very inefficient. This is due to the delivery methods for the CRISPR-Cas9 machinery, which include electroporation of CRISPR/Cas9 DNA, mRNA or ribonucleoproteins containing the Cas9-gRNA complex. However, these procedures are quite toxic for the organoids. Here, we describe the use of the 'nanoblade' technology, consisting in virus like particles which incorporate Cas9 protein and its associated guide RNAs. These delivery tools outperformed by far gene editing levels achieved to date for murine and human tissue-derived organoids. We reached up to 75% of reporter gene knock-out in organoids after treatment with nanoblades. Indeed, high-level nanoblade-mediated knock-out for the androgen receptor encoding gene and the cystic fibrosis transmembrane conductance (CFTR) regulator gene was achieved with single gRNA or dual gRNA containing nanoblades in murine prostate and colon organoids. Likewise, nanoblades achieved 20% to 50% gene editing in human prostate and colon organoids. Only a five minute incubation was required to achieve highly efficient knock-out of human CFTR in human colon organoids. Most importantly, in contrast to other gene editing methods, this was obtained without toxicity for the organoids confirmed by unchanged organoid counts or sizes. Only four weeks are required to obtain stable gene knock-out in organoids. summarizing, nanoblades simplify and allow rapid genome editing in organoids with little to no side-effects including unwanted insertion/deletions in off-target sites thanks to transient Cas9/RNP expression.

INV58

Harnessing mobile genetic elements to write DNA sequences with RNA

M C Holmes [1]
1: Tessera Therapeutics

The ability to introduce new DNA sequence into the genome with high specificity and efficiency would provide a critical complement to existing gene editing approaches. Naturally occurring mobile genetic elements, such as transposases, retrotransposases, and recombinases, offer a rich reservoir of activities that can be exploited to introduce genetic information. These highly abundant, and functionally diverse elements vary in their nucleic acid sequence, biochemical mechanism of substrate integration, and the efficiency and specificity by which they integrate their substrate. We have identified, prioritized, and tested thousands of previously uncharacterized mobile genetic elements for their ability to mediate the introduction of genes into the human genome. We conducted a systematic in silico analysis of tens of thousands of

naturally occurring mobile genetic elements from across the tree of life and developed screening strategies to evaluate the activity of these newly characterized enzymes in human cells. We further engineered these elements to generate synthetic molecules that enhance and expand their natural activities. We refer to these engineered mobile genetic elements as either RNA Gene Writers or DNA Gene Writers, depending on their use of an RNA or DNA template to “write” new sequence into the genome. This screening paradigm and further high throughput engineering resulted in the development of several enzymes that can catalyze a broad spectrum of editing reactions from introduction of large DNA sequences to small single nucleotide changes. Here we will discuss our screening and engineering platform to develop RNA Gene Writer systems that enable this full spectrum of edits by delivery of all-RNA components to primary cells in vitro and in vivo. We will present on RNA Gene Writers that can efficiently engineer primary human T cells to express a functional Chimeric Antigen Receptor (CAR) and promote tumor cell killing both in vitro and in vivo. Moreover, we will present the application of our RNA Gene Writers to edit the phenylalanine hydroxylase (PAH) gene in mice to correct two disease models of Phenylketonuria (PKU) and in cynomolgus monkeys to install a surrogate nucleotide change at efficiencies well above the threshold required for phenotypic rescue in disease models

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INV59

How to engineer human pluripotent stem cells to understand human development and disease

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1: Catalan Institution for Research and Advanced Studies, Institute for Bioengineering of Catalonia, Barcelona

In recent years considerable progress has been made in the development of faithful procedures for the differentiation of human pluripotent stem cells (hPSCs). An important step in this direction has also been the derivation of three-dimensional cell cultures that represent micrometer to centimeter size versions of human organs, the so-called organoids.

Here we will discuss current developments in the hPSCs-organoid field and emphasize the achievements and ongoing challenges of bringing together hPSC organoid differentiation, bioengineering and disease modelling with a particular focus on genetic and systemic disorders compromising kidney. We will further discuss on how the convergence of stem cell biology and bioengineering now offers the possibility to provide physiologically relevant stimuli in a controlled fashion in these model systems resulting in the development of naturally inspired approaches to overcome major limitations of the organoid field.

INV60

Drug discovery of mitochondrial disorders with engineered stem cells and brain organoids

A Prigione [1]
1: University of Dusseldorf

Energy metabolism is essential for providing the energy necessary to ensure proper cellular function. Mutations in genes regulating this process lead to inherited metabolic disorders that can particularly affect tissues with high energy demands like the brain. Among incurable inherited metabolic diseases, mitochondrial diseases represent a major therapeutic challenges, as that they can be caused by mutations in genes that are encoded by either the mitochondrial DNA (mtDNA) or the nuclear DNA (nDNA). Given the challenges associated with mtDNA engineering, there is a lack of effective model systems for screening and testing drugs.

In this talk, I will summarize our efforts in using patient-derived and engineered induced pluripotent stem cells (iPSCs) to study mitochondrial diseases. We focus primarily on Leigh syndrome, which is the most frequent and most severe mitochondrial disease affecting 1/40,000 newborns. We show that neuronal cultures and brain organoids derived from Leigh syndrome iPSCs can be used as model systems to investigate the neuropathological mechanisms and to carry out phenotypic compound screenings. Our data pave the way to the identification of disease-modifying therapies for currently incurable mitochondrial disorders.

INV61

Engineered CRISPR Technologies to improve genome editing

B Kleinstiver [1]
1: Mass General Hospital & Harvard Medical School, Boston, MA

User-specifiable genome modifications are now possible by using gene editing approaches. Despite the vast potential of CRISPR technologies for editing genomes, many properties of Cas enzymes remain suboptimal for laboratory or therapeutic use. We are therefore deploying protein engineering strategies to improve the intrinsic properties of CRISPR enzymes, including their ability to access the genome efficiently and safely (with high precision and accuracy). Our recently developed Cas enzyme, named SpRY, offers unparalleled access to the genome for various applications 1,2 . We are exploring the utility of engineered Cas enzymes as base editors in preclinical experiments to treat several monogenic disorders (including spinal muscular atrophy 3 and others). Despite our observations of high levels of in vivo editing and robust phenotypic benefits, for most diseases, the necessity of developing bespoke editing strategies for each patient-specific mutation imposes challenges that impact the scalability of these approaches 4 . Therefore, we are also developing CRISPR technologies capable of larger kilobase-scale edits. We recently improved the natural properties of CRISPR-associated transposases (CASTs), which are capable of programmable RNA-guided genomic integration of DNA cargos 5.

Finally, we are developing other genome editing approaches that leverage the advantageous properties of DNA-dependent DNA polymerases (DDPs) to install nearly any short, medium, or large sized DNA edit 6 . These new technologies, termed 'click editors', utilize simple DNA oligonucleotides tethered to a nCas9-bound target site as a template for genome writing (permitting the installation of edits encoded on the template DNA molecule). Together, these engineered enzymes offer new capabilities for generating small and large genetics edits, simplifying editing strategies towards the development of new tools and genomic medicines.

INV62

Epitope Engineering for an Immunotherapy "Stealth" Hematopoiesis

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1: Boston Children's Hospital 2: Dana-Farber Cancer Institute 3: Harvard Medical School 4: University of Milan

Despite the considerable efficacy observed when targeting a dispensable lineage antigen, such as CD19 in B cell acute lymphoblastic leukaemia, the broader applicability of adoptive immunotherapies is hampered by the absence of tumour-restricted antigens3–5. Acute myeloid leukaemia immunotherapies target genes expressed by haematopoietic stem/progenitor cells (HSPCs) or differentiated myeloid cells, resulting in intolerable on-target/off-tumour toxicity. Here we show that epitope engineering of donor HSPCs used for bone marrow transplantation endows haematopoietic lineages with selective resistance to chimeric antigen receptor (CAR) T cells or monoclonal antibodies, without affecting protein function or regulation. This strategy enables the targeting of genes that are essential for leukaemia survival regardless of shared expression on HSPCs, reducing the risk of tumour immune escape. By performing epitope mapping and library screenings, we identified amino acid changes that abrogate the binding of therapeutic monoclonal antibodies targeting FLT3, CD123 and KIT, and optimized a base-editing approach to introduce them into CD34+ HSPCs, which retain long-term engraftment and multilineage differentiation ability. After CAR T cell treatment, we confirmed resistance of epitope-edited haematopoiesis and concomitant eradication of patient-derived acute myeloid leukaemia xenografts. Furthermore, we show that multiplex epitope engineering of HSPCs is feasible and enables more effective immunotherapies against multiple targets without incurring overlapping off-tumour toxicities. We envision that this approach will provide opportunities to treat relapsed/refractory acute myeloid leukaemia and enable safer non-genotoxic conditioning.

INV63

Advances in In Vivo CRISPR therapeutics

L Sepp-Lorenzino [1]
1: Intellia Therapeutics

NTLA-2001 is an investigational CRISPR-based therapy with the potential to be the first single-dose treatment for ATTR amyloidosis. NTLA-2001 is being evaluated in a Phase 1, two-part,

open-label study in adults with hereditary transthyretin amyloidosis with polyneuropathy (ATTRv-PN) or transthyretin amyloidosis with cardiomyopathy (ATTR-CM). Clinical data from both study arms will be discussed.

NTLA-2002 is Intellia's *in vivo* investigational drug candidate for hereditary angioedema (HAE), designed to knock out the KLKB1 gene in the liver with the potential to permanently reduce total plasma kallikrein protein and activity, a key mediator of the disease. This investigational approach aims to prevent attacks for people living with HAE by providing continuous reduction of plasma kallikrein activity, following a single dose, and to eliminate the significant treatment burden associated with currently available HAE therapies. NTLA-2002 is being evaluated in a Phase 1/2 study in adults with Type I or Type II HAE. Clinical data including safety, kallikrein reduction and HAE attack rate data will be discussed.

INV65

Update on US phase I/II clinical trial of AMT-130 gene therapy for the treatment of Huntington's disease

R Porter [1]
1: uniQure

AMT-130 is an investigational, modified-AAV5 viral vector containing an exon-1 HTT-targeting miRNA, thereby lowering total-HTT mRNA. This presentation will describe interim 12 and 24-month results from high- and low-dose cohort participants, respectively, from the ongoing HD-GeneTRX 1 trial (NCT04120493). For this analysis, 26 patients, 10 low-dose (6 treated, 4 control) in cohort-1 and 16 high-dose (10 treated, 6 control) in cohort-2, are included. Baseline means (range) for low-dose were: age 49.5 (44-57), CAG repeat length 42.2 (41-44), CAP score 417.7 (322.9-485.9), TFC 12.0 (11-13), TMS 14.5 (8-23), and cUHDRS 14.7 (11.5-18.28). For high-dose, these were: age 47.8 (33-65), CAG repeat length 41.8 (40-46), CAP score 380.2 (278.9-495.0), TFC 11.9 (9-13), TMS 13.9 (6-26), and cUHDRS 13.9 (6-26). Most common adverse events (AEs) for treated cohorts were related to the surgical procedure: procedural headaches, complications, and pain. Two serious AEs which have since resolved, severe headache and CNS inflammation, occurred in the high-dose cohort. Compared to baseline, clinical function was generally preserved at 24 and 12-months for low-dose and high-dose, respectively. As expected, CSF-NfL transiently increased non-dose dependently in all treated participants, peaking approximately 1-month post-surgery before decreasing below and approaching baseline for low-dose and high-dose, respectively. The mHTT data showed some reduction in treated cohorts, in an overall variable data set. Reduction was more pronounced in low-dose than high-dose. Total whole brain volume did not reduce significantly in treated cohorts compared to control. In conclusion, AMT-130 was generally well-tolerated across both cohorts with a manageable safety profile and encouraging trends in clinical and biomarker outcomes.

INV66

Gene therapy strategies for modelling Parkinson's disease in non-human primates

J L Lanciego [1 2]
1: CNS Gene Therapy Program, Center for Applied Medical Research (CIMA), University of Navarra
2: Aligning Science Across Parkinson's (ASAP) Collaborative Research Network

One of the main bottlenecks in drug development is represented by the lack of reliable animal models for end-stage testing of novel therapeutics. Most of the currently available animal models for Parkinson's disease (either transgenic, non-transgenic or neurotoxic) do not reproduce the disease phenotype adequately, therefore novel compounds always failed in early phases when entering clinical trials. At present, there is a continuous race for the development of novel AAV capsid variants with a number of improved properties, such as BBB penetrance, transduction levels, circuit-specific spread through brain circuits, etc. By taking advantage of these newly-available technologies, a wide array of animal models of Parkinson's disease mimicking the known neuropathological hallmarks of this neurodegenerative disorder with unprecedented accuracy has been made available recently, therefore opening new possibilities for a better design of pre-clinical trials, ultimately intended to enhance the chances of success for new drugs when entering clinical trials. Here we will summarize our current expertise in setting-up several different AAV-based animal models of Parkinson's disease, in particular those based in non-human primates.

INV67

Uncovering upsides and pitfalls of base and prime editing in hematopoietic stem cells

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1: San Raffaele Telethon Institute for Gene Therapy (HSR-TIGET) 2: Vita-Salute San Raffaele University 3: National Research Council, Institute for Biomedical Technologies 4: Authors share first authorship 5: Authors share second authorship

Emerging base and prime editing (BE/PE) may provide safer and more precise genetic engineering than nuclease-based approaches bypassing the dependence on DNA double strand breaks (DSBs). However, comprehensive characterization of efficiency, tolerability and genotoxicity of these platforms is lacking and relevant to instruct safe clinical application. Here, we comparatively assessed state-of-the-art cytidine and adenine BE, such as BE4max and ABE8.20-m respectively, and PE versus Cas9 in human hematopoietic stem/progenitor cells (HSPCs) upon mRNA delivery. When targeting a common locus using the same sgRNA, ABE8.20-m outperformed BE4max and Cas9 reaching up to 90% of alleles modification. Cas9-edited HSPCs displayed the lowest engraftment capacity, despite all treatments maintained multilineage reconstitution and no output skewing, as assessed by clonal tracking analyses. Cas9 editing, however, showed moderate shrinkage of clonal complexity in xenotransplanted mice. However, BE4max edited cells tended to decrease over time in the graft pointing to some detrimental response to the treatment in the long-term engrafting HSPC subset. Transcriptional analyses uncovered that BE4max trigger p53

pathway activation, albeit to lower extent as compared to Cas9 treatment, likely due to the conversion of nickase-induced DNA single-SB to DSB. Indeed, sequencing of the target locus revealed that DNA DSBs were less frequent but not abrogated by BE, particularly for the cytidine BE due to suboptimal inhibition of base excision repair. Concordantly, large deletions and translocations were detected upon BE4max treatment despite to lower frequency than upon Cas9 editing. Moreover, ultrahigh coverage whole exome sequencing on long-term xenografts uncovered a genome-wide effect of BE4max on the mutational landscape of hematopoietic clonotypes. Conversely, we did not observe detectable impact of ABE8.20-m in the same analysis. Additionally, both BEs, but not Cas9, upregulated interferon-stimulated genes, suggesting sensing of exogenous long mRNAs. mRNA engineering abolished interferon response without aggravating p53 activation and allowed >80% editing for all platforms in long-term xenografts. In addition, tailored BE4max expression resulted in more efficient BER inhibition leading to reduced proportion of DSBs and improved precision at the target site. We then applied the same mRNA engineering strategy to PE and reached >50% editing in HSPCs. Around 10% of PE alleles carried imprecise editing outcomes, including indels and long-range deletions, despite less frequently than upon Cas9 treatment. Transcriptional analyses showed upregulation of p53- and apoptosis-related genes upon PE. While some p53 targets were shared by PE and Cas9 treatments, others were specific of PE and likely due to cellular sensing of the reverse transcriptase. Despite these adverse effects reduced the hematopoietic graft size compared to mock electroporated controls, >50% PE efficiency was maintained upon serial transplantation. Overall, our study validated the potential for precise and effective editing of adenine BE among emerging nickase-based platforms, building confidence on its current entry into the clinical arena.

INV68

Genome editing strategies for beta-hemoglobinopathies

A Miccio [1]
1: Imagine Institut

Beta-hemoglobinopathies are caused by mutations affecting adult hemoglobin β-chain production. The only curative treatment is allogenic hematopoietic stem/progenitor cells (HSPCs) transplantation, an approach limited by compatible donor availability and immunological complications. Therefore, transplantation of autologous, genetically modified HSPCs is an attractive therapeutic option. However, current gene therapy strategies based on the use of lentiviral vectors or CRISPR/Cas9 nuclease are not equally effective in all the patients and/or raise safety concerns. Base editing and prime editing are novel, promising CRISPR/Cas9-based genome editing technologies that allow the introduction of mutations in the DNA without generating dangerous double strand breaks. Dr. Miccio will discuss the different genome editing strategies aiming either to correct the genetic defect or target disease modifiers in patient cells and provide a safe and effective treatment for β-hemoglobinopathies.

INV69

Lentiviral mediated gene therapy for artemis-deficient severe combined immunodeficiency

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1: UCSF Benioff Children's Hospital

Artemis-deficient SCID (ART-SCID) is an ultrarare inborn error of immunity. It is estimated that 2-3 babies are born with ART-SCID in the U.S. annually. Artemis is a nuclease with both endo- and exonuclease activities that is important in double stranded DNA break repair and essential for V(D)J recombination and the development of T and B cell receptor diversity. Patients with ART-SCID are unable to make T and B cells in addition to having increased sensitivity to ionizing radiation and alkylating agents. ART-SCID is the most difficult type of SCID to treat with an allogeneic hematopoietic cell transplant (HCT) due to increased rejection, poor immune reconstitution, increased GVHD, and poor overall survival. For these reasons we initiated a first-in-human clinical trial of lentivirus-mediated gene insertion therapy into autologous hematopoietic stem cells for patients with ART-SCID. Since 2018 we have enrolled and treated 13 newly diagnosed children with ART-SCID. Overall survival (OS) is 100% with event free survival (EFS) of 90%. Most patients (90%) followed for at least 24 months have reconstituted T cell immunity and 60% have normal B cell immunity to date. Immune reconstitution, OS and EFS are superior in the gene therapy cohort compared to an historical control cohort treated with alternative donor allogeneic HCT.

INV70

Universal Survival and Superior Immune Reconstitution after Lentiviral Gene Therapy with Low Dose Conditioning for X-linked SCID (SCID-X1)

C Booth[1]
1: Great Ormond Street Institute of Child Health, London

Introduction: Boys with X-linked severe combined immunodeficiency (SCID-X1) lack T and NK cells. B cells fail to function due to lack of IL-2 receptor gamma (IL2RG) signaling. In SCID-X1, corrected progenitors can engraft in the thymus and give rise to mature T cells, without chemotherapy conditioning. After allogeneic transplant (HCT), ~50% of patients achieve optimal T cell recovery at 6 months (PMC6202916, 2018). Trials of gene therapy (GT) using a gammaretroviral (gRV) vector led to T cell development but also insertional oncogenesis. Deletion of gRV LTR enhancers generating a self-inactivating gRV (SIN-gRV) vector led to similar T cell recovery with no leukemias to date (PMC4274995, 2014). Building on this success, we opened a multi-institutional international trial using a lentiviral (LV) vector with codon optimized IL2RG, the same cis-regulatory elements as in the SIN-gRV trial, and busulfan conditioning (NCT03311503/03601286). Our goal was to achieve rapid T and NK cell reconstitution, and B cell function that would be superior to HCT and the previous SIN-gRV trial. **Methods:** Of 15 enrolled, 13 successfully underwent CD34+ HSC manufacturing at median age 3.5m (1.8-5.6), using mobilized peripheral blood in 12/13 and transduction enhancers in 11/13. Median CD34+

cell dose/kg and vector copy number (VCN) were 9.8 x 10e6 (4.7-17.87) and 1.1 copies/diploid genome (dg) (0.67-3.07). Thawed drug products (DP) were infused after busulfan targeted to AUC 30 mg*h/L given over 2 days at median age 5.2 months (3.2-7.1). **Results:** All 13 are alive with median follow-up 2.6y (0.2-4.9) with no treatment-related serious adverse events or clonal expansions. All subjects ≥6m post-GT had superior T cell recovery compared to standard HCT (CD3+ T cell >1000 9/9 vs 67/136, p=0.009; CD4+ T cell >500 9/9 vs 72/135, p=0.02). Compared to the SIN-gRV trial, CD3+ and CD4+ T cell numbers were higher at 6m post-GT in conditioned SIN-LV subjects, indicating more rapid recovery (p=0.0002). Sustained multilineage gene marking was seen in B, NK cells and neutrophils. In contrast to SIN-gRV recipients treated without conditioning who remain on IgG replacement therapy (IgRT), 7/8 SIN-LV subjects >1y post-GT are off IgRT; 5/5 responded to vaccines. CD3, CD4 and NK cells were higher at 2y post-GT compared to unconditioned SIN-gRV recipients (p=0.007, 0.002, 0.003). VCN in T, B, NK, and neutrophils was higher in those receiving DP with VCN ≥2 compared to VCN <2 copies/dg (p=0.004, 0.02, 0.02, 0.02). **Conclusion:** GT using a LV with codon optimized IL2RG and busulfan conditioning resulted in 100% survival and robust T cell recovery more rapid than after standard HCT. Conditioning was associated with sustained multilineage gene marking and B cell function.

INV71

Building flexible end-to-end cell therapy manufacturing strategies

G Vallanti [1]
1: AGC Biologics, Milan

Cell therapy processes are highly variable in terms of cell type, length, volumes, reagents, equipment and testing considering both autologous and allogenic setting and processes for cell modification or only cell expansion.

A CDMO as AGC Bio willing to compete in this highly complex and variable environment need to have flexibility in each critical steps, from high specialized technical capabilities, equipment base, facility design, analytical platform to reduce the time to GMP and optimize throughput .
During the presentations we will navigate through different processes aspects and facility design with concrete examples of processes running in AGC bio facility as real case studies.

INV72

Breaking the myths of challenging large scale AAV manufacturing

H Lesch [1]
1: Exothera, Brussels

Recombinant adeno-associated viruses (AAVs) are widely used as primary viral vectors for gene therapies due to their safety profile, lasting transgene expression, and ease of achieving substantial titers even at a small scale. However, a major hurdle in advancing AAV-based therapies to clinical use lies in the effort required for process development and the efficient

translation of laboratory-scale processes to large-scale Good Manufacturing Practice (GMP)-compliant production while meeting high-quality and dosage demands. A significant investment was made in planning a CMC regulatory strategy and developing the standardized AAV baseline manufacturing platform supported by extensive analytical characterization. The design of experiment (DoE) approach was used to optimize critical parameters and accelerate while scaling up to 1000 L was performed to meet high dosage demand. In our endeavour, we utilized a recombinant AAV8 vector encoding the factor IX protein. We conducted the AAV8 production process at a 40L scale within an Eppendorf BioBLU® 50c Single-Use Bioreactor and executed the process scale-up to 200L and finally to 1000L in Pall Allegro STRs (Stirred Tank Reactor), consistently achieving remarkable AAV8 titers, resulting in $8.2x10^{11}$ vg/ml titer at 1000L scale. This is a success story of collaboration with Alexion and PolyPlus, showing how we executed right-first-time the scale up project, successfully completing transfer to manufacturing scale in just 9 months. The upstream part was followed by effective downstream development focusing on impurity removal, high step recovery and quality improvement, especially empty/full particles separation. In summary, standardized viral vector manufacturing platforms ensure product quality, regulatory compliance, reduced costs and accelerated development and production of viral-based therapies on a large scale.

INV73

Meeting the moment: Overcoming manufacturing bottlenecks and technical challenges to usher a new era of genetic medicines

N Loggia [1]
1: Orchard Therapeutics

Hematopoietic stem cell (HSC) gene therapy holds immense promise for the treatment of a wide range of diseases and conditions. Unlike traditional pharmaceuticals, these therapies harness the power of a patient's own HSCs, offering a personalized approach to medicine. Despite the clinical utility and promise, the translation of these therapies from the laboratory to large-scale manufacturing faces challenges that must be overcome to unlock the full potential of this groundbreaking technology. Critical bottlenecks lie in the production of lentiviruses for the gene modification as well as largely manual cell processing methods making it challenging to scale up production and meet the growing demand for these therapies. Moreover, establishing a robust end-to-end product traceability remains a complex logistical challenge to ensuring these innovative therapies reach the patients who need them. The presentation will provide valuable insights into novel approaches to optimizing the production and supply to break down these barriers and usher in a new era of personalized medicine, where autologous therapies are readily available to all who need them.

INV75

Early phase trials of CRISPR/Cas9 and base-edited CAR T cells

W Qasim [1]
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The introduction of genome editing steps into the production of chimeric antigen receptor (CAR) T cells is providing new therapeutic strategies and generating early phase human data. Overcoming HLA barriers to allow allogeneic donor derived CAR T cell banks to be used where autologous products are not suitable or available is of particular interest. Transcription activator-like effector nucleases (TALENs) were initially used to generate 'universal' CAR19 T cells that were used without HLA matching to treat relapsed B cell acute lymphoblastic leukaemia (B-ALL) by disrupting T cell receptor expression after lentiviral transduction, and rendering cells insensitive to Alemtuzumab by CD52 knockout. Anti-leukemic effects were sufficient to secure clearance within four weeks, and provided a remission-bridge to transplantation. Subsequently, lentiviral delivery of CAR19 was coupled to multiplexed LTR-embedded CRISPR guides for a refined version of the approach which was piloted in a trial for paediatric patients unsuitable for autologous CAR19. Similar anti-leukemic responses and toxicity profiles were recorded, with children achieving remission by day 28, then proceeding to allo-SCT. The approach has been developed further to allow CAR T approaches to address relapsed T-ALL and acute myeloid (AML) with additional knockouts incorporated to remove shared antigens that would otherwise mediate fratricide effects. These latest iterations used cytidine deamination mediated base-editing for triple gene knockouts through the creation of premature stop codons or disruption of splice sites rather than double stranded DNA breaks and repair elicited by nuclease editors. Phase I trials of base edited CAR7 T cells are underway, with early evidence of successful responses in paediatric T-ALL.

INV77

Considerations in the development of an investigational gene therapy for otoferlin gene-mediated hearing loss

M Simmons [1]
1: Akouos

Millions of individuals worldwide have disabling hearing loss because one or their genes generates an incorrect version of some specific protein the ear requires for hearing. In many of these cases, delivering the healthy version of the gene to a target cell within the inner ear has the potential to restore auditory function to enable high-acuity physiologic hearing. Children with mutations in the otoferlin gene (*OTOF*) typically present with congenital, Severe to Profound sensorineural hearing loss with absent or reduced auditory brainstem responses and present otoacoustic emissions, consistent with auditory neuropathy. We outline our strategy for the development of a genetic medicine with the potential to restore hearing in individuals with *OTOF*-mediated hearing loss.

INV78

In silico risk assessment of T cell response to micro-dystrophin in DMD patients harboring mutations in dystrophin N-terminal region and treated with AAV-based gene therapies

A Hollenstein [1] S Khan [3] R Xicluna [1] D Sauter [1] A Ducret [1] G Steiner [1] I Moeller [3] S Mason [3] E Darton [3] R Potter [3] P Veron [4] S Braun [4] M Guridi Ormazabal [1] A Murphy [2] C Wandel [1] H Haegel [1]
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Duchenne muscular dystrophy (DMD) is a rare, X-linked neuromuscular disease caused by mutations in the *DMD* gene leading to an absence of functional dystrophin. Adeno-associated virus (AAV) vector-based gene therapies have been designed to transduce muscle cells, delivering transgenes encoding for shortened, functional versions of dystrophin, called micro-dystrophin. Due to its low homology to utrophin or to other human proteins, the N-terminal region of micro-dystrophin is thought to carry a particular risk of immunogenicity following AAV-mediated gene transfer in DMD patients harboring mutations in dystrophin N-terminal region. In clinical trials using a micro-dystrophin transgene covering the N-terminal and C-terminal regions of dystrophin, Suspected Unexpected Severe Adverse Reactions (SUSARs) occurred in the form of immune-mediated myositis. These patients had dystrophin mutations affecting the N-terminal region and showed strong IFN-g responses to this domain of the protein in ELISpot assays, suggestive of a cytotoxic T cell response.

Patients with mutations preventing the native expression of dystrophin sequences bear the risk of epitopes in the transgene product being recognized as foreign and in turn eliciting an immune response. One prerequisite for detection by the immune system is the presentation of peptide fragments of the transgene product by HLA class-I and class-II molecules, respectively driving $CD8^+$ and $CD4^+$ T cell responses. The HLA-I and -II alleles were genotyped in patients who had mutations affecting the dystrophin N-terminal region and who either had a SUSAR or were safely dosed. Using the *in silico* tool NetMHCpan, we aimed to assess the propensity of micro-dystrophin peptides to bind HLA-I or HLA-II molecules, for each patient's HLA allele combinations. For each of the N-terminal exons, epitope scores were calculated based on the number of predicted HLA-binding peptides. Results showed a strong HLA-dependent variability, but highest epitope scores were obtained for exons 8 and 9 corresponding to the Hinge 1 domain of dystrophin. Moreover, using MHC-Associated Peptide Proteomics (MAPPs) performed in a range of healthy blood donors, HLA-I and -II associated epitopes frequently mapped to this domain.

Since patients must be immunologically tolerant to the sequences encoded by exons that are not affected by their particular mutations (recognized as "self" sequences), epitope scores were considered only for the exons corresponding to the deleted / altered protein sequences ("non-self"). With this analysis, the SUSAR patients were found to bear higher risks of developing a HLA-I dependent immune response to peptides encoded by exons 8 and 9. These findings were supported by the identification of T cell epitopes for the SUSAR patients in ELISpot assays, showing IFN-g responses directed against peptides mapping to this region.

These results point to the immunogenicity risk that can be associated with micro-dystrophin gene transfer in patients deleted of dystrophin exons 8 and 9.

INV79

Addressing AAV vector immunogenicity in humans

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1: Spark Therapeutics

In vivo gene therapy offers several potential advantages over conventional therapeutic modalities. These include the long-term duration of effect after a single intervention, the ability to target genetic defects and pathways by directly expressing a transgene of interest, and the possibility of delivering a therapeutic gene directly in the tissue/cell where it is needed. Thus far, the most broadly adopted platform for in vivo gene therapy is based on adeno-associated virus (AAV) vectors, which are viral vector derived by viruses isolated in nature or engineered to achieve a desired tissue tropism. Several preclinical and clinical trials have shown that AAV vectors have a favorable safety and efficacy profile, and regulatory approval was obtained for gene therapies for several genetic diseases. Emerging long-term follow up data are also encouraging, showing multi-year efficacy in most cases. Immunogenicity of AAV still represents an important obstacle to the successful translation of AAV gene therapies to humans, and consequently, to a broader use of the platform in drug development. As humans are naturally exposed to wild-type AAV, they develop cross-reactive immunity to the gene therapy vector. Similarly, the human immune system is equipped to react to the administration of AAV vectors in a similar was it reacts to viral infections. In most cases, immune responses to AAV vectors have resulted in either lack of efficacy (due to antibody-mediated neutralization) or loss of transgene expression with asymptomatic and self-limiting increase in liver enzymes. However, high doses of AAV vectors administered systemically have resulted in severe adverse reactions in some cases associated with complement activation. Inflammation has also been reported in trials in which AAV vectors were used to target the eye or the central nervous system. Immunomodulatory regimens, targeted to specific pathway driving vector immunogenicity, and strategy to address humoral immunity to AAV, are in development. Similarly, the development of AAV vectors with improved tropism, and enhanced design of expression cassettes, along with enhanced vector manufacturing technologies, will help unlocking the full potential of in vivo gene therapy.

INV81

Danon Disease phase 1 RP-A501 results: the first single-dose intravenous (IV) gene therapy with recombinant adeno-associated virus (AAV9:LAMP2B) for a monogenic cardiomyopathy

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Danon disease (DD) is a rare X-linked monogenic cardiomyopathy and multisystemic disorder caused by *LAMP2* gene mutations resulting in LAMP2 protein deficiency. Male DD patients (pts) develop severe progressive hypertrophic cardiomyopathy (HCM), left ventricular (LV) dysfunction

and arrhythmias resulting in mortality at a median age of 19-20 years (y). This open-label, single-dose, phase 1 trial enrolled male DD pts with pathogenic *LAMP2* mutations and HCM in two age groups: ≥15 y (n=5) and 8-14 y (n=2). Pts received IV infusion of RP-A501, an adeno-associated virus serotype 9 encoding a normal copy of the human *LAMP2B* isoform (AAV9.LAMP2B) at 6.7 x 10^{13} GC/kg (low dose) or 1.1 x 10^{14} GC/kg (high dose). Transient immunomodulation (IM) included prednisone, rituximab, and sirolimus (for the most recently treated pediatric pts). Between June 2019 and March 2022, 7 males (5 adult, 2 pediatric) with DD age 11.7 - 21.1y (median 18.3y) received RP-A501 (N=5 low dose and N=2 high dose). All pts were NYHA Class II at baseline. IM compliance was confirmed in 6/7 pts. All pts are alive and stable at 36 months (m) follow-up. One adult pt with baseline LV systolic dysfunction had progressive heart failure likely related to DD progression and required heart transplant at 5m post RP-A501. Follow-up for the 6 pts with baseline LVEF >40% was 24-36 months (adult pts) and 6-12 months (pediatric pts). All RP-A501- or IM-related adverse events (AEs) were manageable and reversible. A single RP-A501-related SAE of grade 4 thrombotic microangiopathy and renal failure requiring transient dialysis (with full recovery from both) was reported in 1 adult pt in the high dose cohort. No RP-A501 or steroid-related SAEs have been observed to date in the pediatric pts. On baseline endomyocardial biopsy, LAMP2 expression was grade 0 (negative staining by immunohistochemistry) in all patients; 100% (N=6/6) of evaluable pts had cardiac LAMP2B transgene expression within 6m of therapy with reduced LV mass (14-48% decrease from baseline) per echocardiogram. Pts evaluable beyond 6m had stabilized or improved BNP (N=6/6) and troponin (N=6/6) by 6-12m, and stabilized/improved LV wall thickness (N=5/5) and NYHA Class (N=5/5) by 12-18m. These findings persist up to 36m post RP-A501. Improvements/stabilization in BNP diverge markedly from natural history, including those observed in an ongoing prospective study (NHS, UCSD: NCT03766386). In untreated patients of similar age, NPs increased over 3-30m. In this prospective NHS, no patient had improved NYHA class over 6-24 months, in contrast to the NYHA improvements observed following RP-A501. Results from this phase 1 trial in DD demonstrate that B- and T-lymphocyte directed immunomodulation enables safe IV RP-A501 gene therapy administration, resulting in cardiomyocyte transduction, LAMP2B expression, improved autophagy, and improved/stabilized serologic, echocardiographic and clinical parameters associated with prognosis in HCM. These data support activation of a phase 2 clinical trial utilizing the 6.7 x 10^{13} GC/kg (low) dose. Updated results will be presented.

INV82

RNA therapies for cardiac regeneration and precise gene editing

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Cardiac disorders are common, lethal and expensive. Heart failure, which is a common consequence of most cardiac conditions, including cardiomyopathy of ischemic or hereditary origin, now affects over 1-3% of the global adult population, has a 5-year mortality of 50-75% and absorbs 2-3% of national health expenditures in high-income countries. Cardiomyocyte death is not offset by new cell generation after birth, as the regenerative capacity of the adult human myocardium is less than 1% per year over a lifetime. No drug exists for myocardial survival or regeneration, nor any gene editing therapy for the correction of genetic defects. More in general, no biological therapy has ever been developed for any primary cardiac condition.

A main goal of my laboratory is to develop RNA therapeutics to treat cardiac disease, with the objectives of preventing cardiomyocyte loss after damage, stimulating cardiac regeneration and

achieving precise gene editing for the correction of hereditary cardiac mutations. Using whole genome siRNA and miRNA libraries, over the last few years we performed a series of high throughput screenings for non coding RNAs that prevent cardiomyoycyte death, stimulate cardiomyocyte proliferation and promote homology directed repair and prime editing using CRISPR/Cas9 tools. We have identified a few miRNAs that are very effective at stimulating re-entry of cardiomyocytes into the cell cycle. Once administered to mice or pigs after myocardial infarction, these miRNAs promote clinically relevant cardiac regeneration and remuscularisation of the infarcted hearts. Other miRNAs stimulate precise gene editing by reawakening expression of the homologous recombination machinery in post-mitotic, adult hearts or stimulating prime editing. We achieve expression of these miRNAs either using AAV vectors or by delivering their synthetic mimics to the heart using lipid nanoparticles (LNPs) generated with the SNALP technology, which is amenable to clinical translation in patients.

INV83

Development and approval of oncolytic herpes virus G47Δ for malignant glioma in Japan

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Genetically engineered, conditionally replicating herpes simplex viruses type 1 (HSV-1) are promising therapeutic agents for cancer. A triple-mutated, third-generation oncolytic HSV-1, G47Δ, exhibits enhanced replication capability in a variety of cancer, efficient induction of specific antitumor immunity, and high safety features. G47Δ also kills cancer stem cells efficiently. The investigator-initiated phase II clinical trial of G47Δ in adult patients with recurrent or residual glioblastoma was performed from 2015 to 2020. In this trial, each patient received stereotactic injections with G47Δ (1×10^9 pfu/dose) into the tumor, repeatedly, but into different coordinates, for the maximum 6 times. In most patients, G47Δ was given in addition to the maintenance chemotherapy using temozolomide. The trial was terminated early due to a significantly high efficacy shown at the interim analysis and 19 patients in total were registered. The primary endpoint of 1-year survival rate after G47Δ initiation was 84.2% (16/19). The median overall survival was 20.2 months after G47Δ initiation and 28.8 months from the initial surgery. The most common G47Δ-related adverse event was fever (17/19) followed by vomiting, nausea, lymphocytopenia and leukopenia. On MRI, enlargement of and contrast-enhancement clearing within the target lesion repeatedly occurred after each G47Δ administration, which was characteristic to this therapy. Thus, the best overall response in 2 years was partial response in one patient and stable disease in 18 patients. Biopsies revealed increasing numbers of tumor-infiltrating $CD4^+$/$CD8^+$ lymphocytes and persistent low numbers of $Foxp3^+$ cells. This study showed a survival benefit and good safety profile, which led to the approval of G47Δ for malignant glioma as the first oncolytic virus product in Japan in June 2021. G47Δ is commercially available since November 2021, and now used in clinical practice. The use of G47Δ may become a preferred treatment that potentially leads to a cure of malignant glioma.

INV84

Long term clinical trials for eladocagene exuparvovec, a gene therapy for AADC deficiency

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Eladocagene exuparvovec, the first gene therapy to be surgically delivered directly into brain tissue, was approved in July 2022 by the European Medicines Agency for the treatment of patients with aromatic L-amino acid decarboxylase deficiency (AADCd). AADCd is a rare disease affecting the synthesis of monoaminergic neurotransmitters and many patients exhibit significant motor, cognitive, and autonomic impairments. Current pharmacologic treatment is symptomatic in nature but does not treat the underlying cause of the disease. The delivery of gene therapy directly into the brain ensures gene therapy product reaches the desired target, with lower risk for systemic immunogenicity and exposure.
DDC gene delivery to the putamen, the target site of dopaminergic activity in the nigrostriatal pathway was initially explored for the treatment of Parkinson's disease. Because AADCd deficiency affects the same pathway, *DDC* gene delivery was explored in patients with this disease using the same target site. The first observational compassionate use study of eladocagene exuparvovec began in 2010, with the first patient with AADCd treated in 2011. Since that time, two other studies have been conducted, and as of February 2020, 28 patients were treated across the three studies. The primary efficacy endpoint of the trials was the achievement of key motor milestones (head control, sitting unassisted, standing with support, and walking assisted) by 24 months after gene therapy. Rapid improvements in motor and cognitive function were observed within 12 months following gene therapy and were sustained during follow-up for >5 years. Improvements in patient symptoms (mood, sweating, temperature, and oculogyric crises), body weight and caretaker QoL were also observed. There were no treatment-associated brain injuries, and most AEs were related to the underlying disease. Pyrexia and dyskinesia were the most common TEAEs; most dyskinesia events were transient, mild or moderate in severity and mostly subsided within 7 months. These studies demonstrated the long-term safety and durable efficacy of intraputaminal infusion of eladocagene exuparvovec for patients with AADCd

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INV86

The Bitter Experience - A Thalassaemia Patient's Perspective

G Constandinou [1]
1: Thalassaemia International Federation

Thalassaemia, a group of inherited blood disorders characterized by abnormal haemoglobin production that on many occasions requires long-term regular blood transfusions, remains a global public health concern. This 12-minute presentation will first provide a focused examination of the worldwide impact of thalassaemia, the persistent unmet needs and inequalities, with a particular emphasis on the European context. Key milestones in the history of thalassaemia control will be mentioned, with a particular emphasis on the case of Zynteglo, a groundbreaking gene therapy which currently is inaccessible to patients in Europe.

INV91

Interim skeletal outcome after hematopoietic stem and progenitor cell-gene therapy for mucopolysaccharidosis type I hurler

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Mucopolysaccharidosis type I Hurler variant (MPS-IH) is a rare lysosomal storage disorder caused by defects in the *IDUA* gene and characterized by a wide range of clinical manifestations including a severe and progressive skeletal dysplasia. While allogeneic hematopoietic stem cell transplantation (HSCT) +/- pre-/peri-transplant enzyme replacement therapy represents standard of care for MPSIH, is not able to fully address skeletal manifestations which continue to severely affect patient's quality of life. Autologous hematopoietic stem progenitor cell-gene therapy (HSPC-GT) provides extensive metabolic correction in MPS-IH patients (Gentner et al. N Engl J Med. 2021), however its ability to impact skeletal dysplasia is unknown.

Eight MPS-IH patients (6M, 2F; mean age ±SD at treatment: 1.9±0.5 years) were enrolled in a phase I/II study of HSPC-GT (NCT03488394). Skeletal dysplasia was evaluated in terms of clinical (growth), functional (Peabody scale, joint range of motion [ROM]) and radiological (acetabular index [AI] and migration percentage [MP] at hip X-Rays and MRIs, spine MRI score) parameters at baseline and multiple timepoints up to 4 years after treatment. Specific skeletal measures were compared with an external cohort of HSCT patients.

All patients are alive with sustained engraftment of gene-corrected cells, supraphysiologic blood IDUA and normal/near normal urinary GAG levels. They exhibit longitudinal growth within the normal range with a median height gain greater than that observed in an external cohort of HSCT subjects after 3-year follow-up. After HSPC-GT they experienced a complete and earlier normalization of joint mobility compared with HSCT patients. Mean AI and MP progressively decreased after HSPC-GT, accounting for a reduction in hip dysplasia. Typical spine alterations measured through a specific MRI score showed overall stabilization after HSPC-GT up to 3-year follow-up (mean score: 3.5 at baseline, 3.3 at 3-year follow-up).

This interim skeletal outcome analysis of clinical, functional and radiological parameters indicates stabilization of skeletal dysplasia in MPS-IH patients up to 4 years after HSPC-GT.

Oral Presentations

OR01

Identification of key regulatory factors driving CAR-T cell dysfunction in MM by single cell multiomics

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CAR-T cells have revolutionized cancer immunotherapy, representing a promising option for relapsed/refractory Multiple Myeloma (MM) patients. Nevertheless, despite the high remission rates observed after BCMA CAR-T therapy, a significant number of patients still relapse. However, knowledge of the molecular mechanisms governing CAR-T cell function in MM is very limited. To shed some light on specific transcriptomic programs activated after CAR-T cell administration, we interrogated longitudinal samples of CAR-T cells collected from patients enrolled in ARI0002h clinical trial. In this work we characterized more than 50.000 CAR-T cells from 11 different samples, including infusion products (IP), as well as CAR-T cells isolated from bone marrow (BM) and peripheral blood (PB), at one and three months after infusion. Single-cell RNA and TCR sequencing (scRNAseq, scTRCseq) coupled with SimiC analysis, a novel machine learning algorithm that infers gene regulatory networks (GRNs), were applied to all samples.

scRNAseq revealed that although CAR-T cells from IP presented similar profiles, with highly proliferative CD4+ and CD8+ memory cells, CAR-T cells remaining after infusion were mainly non-proliferating CD8+ cells, with effector/effector-memory phenotypes. Interestingly, transcriptomic profile of CAR-T cells differed among patients, with increased presence of terminally differentiated effector cells presenting an exhausted signature in patients with partial response. In contrast, complete responders presented CAR-T cells in transition to central memory or effector memory phenotype. In addition, CAR-T cells infiltrating BM presented increased

expression of cytotoxic (GZMA, PRF1) and exhaustion (LAG3, HAVCR2, TIGIT) markers compared to their PB counterparts. GRN analysis with SimiC identified several regulons, such as PRDM1 and ARID4B, with increased activity in the CAR-T cells from BM, which could be responsible for these differences. PRDM1 has been already associated with CAR-T cell exhaustion and its depletion promotes TCF7-dependent CAR-T cell stemness and proliferation. ARID4B, a chromatin remodeler TF, could be acting as an epigenetic regulator of CAR-T cell function.

The combination of scTCRseq and scRNAseq allowed the identification of a hyperexpanded CAR-T clone, with immunosuppressor features, mainly present in the BM of a patient with partial response. Deeper characterization showed that this clone had higher expression of cytotoxicity and activation markers, as well as an increased expression of IL10. Further analysis with SimiC showed association of IL10 with transcription factors related to exhausted CD8+ T cells, like CREM, BHLHE40 and again PRDM1, which is also implicated in the production of IL10 in Treg. Additional in vitro studies suggested that subsequent activation of endogenous TCR after CAR T cell activation led to IL10 production, and functional validations corroborated that IL10 reduces CAR-T cell functionality.

Overall, our analysis combining scRNAseq and scTCRseq with novel machine learning models, allowed us not only to characterize transcriptional differences observed between patients and CAR-T localization, but also to identify regulatory mechanisms that could promote CAR-T cell dysfunction and would represent a potential target to be modulated for the development of improved CAR-T therapies for MM.

OR02

CAR ProTcell, towards well-tolerated and persistent off-the-shelf allogeneic CAR-T cells

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Allogeneic chimeric antigen receptor (CAR) T cells pave the way towards off-the-shelf products immediately available for patients and manufactured with cells from selected healthy donors. To avoid graft versus host disease (GvHD) in case of partial human leukocyte antigen (HLA) mismatch between patient and donor, T cell receptor (TCR) expression is knocked down in existing allogenic CAR-T cell therapies. However, it was shown that co-expression of endogenous TCR and CAR leads to superior persistence of T cells and significantly prolonged leukemia control in vivo. Smart Immune is developing a new allogeneic T cell therapy platform based on T cell progenitors, called ProTcell Platform. Human CD34+ cells are cultured for seven days in a clinical grade and feeder-free process using an immobilized fusion protein DLL4-Fc and a cytokine cocktail. In organoids, human ProTcells can differentiate into mature T cells with a naïve & Tscm phenotype. A detailed analysis of ProTcells demonstrated the expression of markers known to be involved in thymic homing such as CXCR4, CCR9. Infused intravenously, ProTcells seed the thymus of NSG mice and differentiate into double positive (CD4+ CD8+) thymocytes and then to CD3+ CD4+ or

CD3+ CD8+ naïve T cells. Despite the presence of TCR, no GvHD was observed after infusion of ProTcells in xenogeneic mouse models and in the first patients treated in phase I trials, thanks to thymic education. Within seven days, CD34+ cells have also been efficiently transduced (>50%) with a lentiviral vector coding for an inducible CAR. Transduced ProTcells can differentiate into mature T cells. CAR T cells obtained from CAR ProTcells can kill target cells in a cytotoxcity assay. We also conduct donor selection algorithm studies as a way of providing HLA match for a reasonable percentage of the target population with a limited number of cell banks. Without the need for any TCR knock down, ProTcells pave the way toward a new generation of off-the-shelf allogeneic CAR T cells, avoiding the risk of GvHD thanks to thymic selection while harboring a functional TCR with the potential to fight infection and cancer relapse. Our algorithm for donor selection will reduce the risk of rejection and increase the likelihood of persistence of this new generation of allogeneic CAR-T cells.

OR03

Cytosine base editor ameliorates the safety profile of TCR edited T cells for the adoptive cell therapy of gastrointestinal tumors

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Adoptive cell therapy (ACT) with T cell receptor (TCR) - transgenic T cells is a promising therapeutic strategy for advanced gastrointestinal cancers. However, the paucity of TCRs targeting relevant tumor antigens and the immunosuppressive tumor microenvironment (TME) limit ACT applicability. We combined the analysis of published datasets with a multidimensional investigation of CRC and PDAC samples collected at Ospedale San Raffaele to select a set of tumor-associated antigens (TAAs) to be targeted by ACT. By clonal tracking of TCR repertoire of T cells stimulated with autologous APCs loaded with the selected antigens, we isolated 6 TCRs specific for different TAAs, including HER2 and mesothelin. TCR edited T cells displayed anti-tumor activity against PDAC cell lines and CRC and PDAC patient-derived organoids while sparing HLA unmatched and antigen negative controls. To boost the efficacy of ACT for solid tumors, an effective tumor recognition needs to be combined with approaches aimed at counteracting the immunosuppressive TME. We identified TIGIT as a key immunosuppressive molecule engaged by PDAC and CRC cells. CRISPR/Cas9 is often selected as a tool to genetically manipulate T cells, however, its activity can produce important off-target events at the genomic level, affecting the safety profile of therapeutic cellular products. To overcome these limitations, we optimized the genome editing approach of T cells by relying on a cytosine base editor (CBE). We generated $HER2_{369\text{-}377}$-specific T cells deprived of the endogenous TCR and TIGIT, a key immunosuppressive molecule, exploiting CRISPR/Cas9 and the cytosine base editor. 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 regions in the genome, mediating sgRNA-dependent and -independent DNA deamination. To assess sgRNA-dependent DNA off-target base editing, we

sequenced the top off-target *loci* for each sgRNA mapping on coding sequences. We confirmed that CBE shows high on-target editing efficiency with minimal off-target activity. To assess sgRNA-independent 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. In functional assays, $TIGIT_{KO}$ TCR edited T cells engineered with CBE displayed superior abilities, compared to TIGIT competent T cells, in eliminating CRC primary and metastatic patient-derived organoids. Our findings suggest that base editors can generate cellular products with a better safety profile and promising therapeutic efficacy in gastrointestinal tumors.

OR04

Significant clinical benefit and enhanced T-cell responses with repeated administration of PRGN-2012, a novel gorilla adenoviral vector based immunotherapy, in adult patients with severe recurrent respiratory papillomatosis

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Recurrent respiratory papillomatosis (RRP) is a rare, neoplastic disorder caused by chronic infection with human papillomavirus (HPV) type 6 or 11. RRP is characterized by growth of papillomas in the upper aerodigestive tract, leading to significant morbidity due to airway obstruction and voice change and infrequently mortality or malignant transformation. There are no approved therapeutics and the current standard-of-care for RRP is frequent ablative surgeries that lead to irreversible laryngotracheal scarring and disability, highlighting the need for medical strategies that address the underlying infection.

PRGN-2012 is a novel, gorilla adenovirus based immunotherapy designed to enhance HPV6/11-specific T cell immunity upon repeated administrations. This is a first-in-human, Phase 1/2 study of PRGN-2012 in patients with severe RRP (NCT04724980). In the Phase 1 portion of the study, patients received 4 subcutaneous injections of PRGN-2012 at dose level 1 ($1x10^{11}$ Particle Units (PU) per injection; n=3) or dose level 2 ($5x10^{11}$ PU; n=12) over 12 weeks. Enrolled patients had severe RRP requiring frequent surgery, with a median total lifetime surgeries of 32 (range 9-300+) and median of 6 (range 3-10) surgeries in the 12-months prior to treatment. PRGN-2012 was well-tolerated at both dose levels, with only mild treatment-related adverse events (TRAEs) which reduced in frequency over the treatment interval. The majority of TRAEs (97%) were Grade 1, and the most common were injection site reaction, fever, chills and fatigue. There were no TRAEs >Grade 2. Based on this safety profile, dose level 2 was selected as the recommended phase 2 dose (RP2D) and was further evaluated in the dose expansion cohort. Treatment at the RP2D resulted in clinically significant benefit with 50% (6/12) of patients experiencing a Complete Response (CR), defined as no interventions required in the 12-months post-treatment with PRGN-2012. All complete responders remain surgery-free as of the data cutoff (follow-up range 440-600 days). 83% (10/12) patients experienced a decrease in the number

of interventions in the 12-months post-treatment compared to the 12-months prior to the start of treatment. PRGN-2012 at the RP2D significantly ($p<0.01$) reduced papilloma burden as quantified by anatomic Derkay scores and significantly ($p<0.01$) improved vocal function as quantified using the Vocal Handicap Index-10, at 24-week follow-up. PRGN-2012 treatment enhanced polyclonal HPV 6/11-specific T-cell immunity in responders whereas anti-drug neutralizing antibodies did not increase with subsequent injections. Additional correlative studies identified features of the papilloma microenvironment related to HPV gene expression, chemokine expression and immune subset infiltration/activity that appear to govern clinical response.

These data demonstrate the overall favorable safety profile and significant clinical benefit of PRGN-2012 in adult patients with severe RRP, with 50% of patients requiring no surgery for a minimum of 12-months post-treatment. There was significant reduction in papilloma burden and concurrent improvement in vocal function post-treatment which was correlated with induction of HPV6/11-specific T-cell immune response. Based on these findings, PRGN-2012 has been granted FDA's Breakthrough Therapy Designation for the treatment of RRP. The Phase 2 portion of the study has completed enrollment and treatment at the RP2D (n=23), and interim data will be presented.

OR05

Global phase 1 study results of lentiviral mediated gene therapy for severe pyruvate kinase deficiency

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Pyruvate kinase deficiency (PKD) is a rare inherited hemolytic anemia caused by *PKLR* gene mutations. Manifestations include anemia, splenomegaly and iron overload, which may be life-threatening. Currently available treatments are limited to a recently-approved enzyme activator or palliative therapies such as chronic blood transfusions, iron chelation therapy and splenectomy which are associated with significant side effects. A global Phase 1 clinical trial RP-L301-0119 (N° EudraCT 2019-001656-1) is underway to evaluate lentiviral mediated hematopoietic stem and progenitor cell (HSPC)-targeted gene therapy for adults and children with severe PKD. Splenectomized patients with severe and/or transfusion-dependent anemia are eligible. Following apheresis, HSPCs are transduced with lentiviral vector carrying the *PKLR* gene and cryopreserved. Myeloablative therapeutic drug monitoring-guided busulfan (target area under the curve [AUC]: 73.1 mg/L*hr) is administered and the gene therapy product (RP-L301) is thawed and infused. Patients are followed for safety assessments (including insertion site analysis [ISA]), and efficacy (genetic correction, decrease in transfusion requirements, significant improvement in

anemia and reduction of hemolysis) for 2 years post-infusion. As of May 3, 2023, 2 adult patients and one pediatric patient have received RP-L301. Patient 1 (adult) received 3.9x10^6 CD34+ cells/kg with mean vector copy number (VCN) of 2.73. Patient 2 (adult) received 2.4x10^6 CD34+ cells/kg with mean VCN of 2.08. Despite baseline hemoglobin (Hb) levels in the 7.0-7.5 g/dL range, both adult patients had normal-range hemoglobin (13.2 g/dL at 24 months post infusion and 14.7 g/dL at 30 months post infusion, respectively), and no red blood cell transfusion requirements post-engraftment. Other parameters of hemolysis and anemia (lactate dehydrogenase [LDH], bilirubin, erythropoietin) are improved. Peripheral blood mononuclear cell (PBMC) vector copy numbers (VCNs) were 1.75 and 1.43 at 24-months. Both patients reported improved quality of life (QOL), also demonstrated by increases in both FACT-An and SF-36 scores, with marked improvement in SF-36 energy/fatigue, physical functioning, and general health components. No serious adverse events (SAEs) have been attributed to RP-L301. Hematopoietic reconstitution occurred within 2 weeks of administration. ISA in PB and BM for both adult patients up to 24 months following therapy demonstrate highly polyclonal patterns and no evidence of insertional mutagenesis; longitudinal results delineating clonal diversity will be presented. Early pediatric data suggests similar clinical efficacy as seen in adult cohort long-term efficacy data. Infusion was well-tolerated in the initial pediatric patient with engraftment achieved at day +15. There were no red blood cell transfusion requirements following engraftment. Clinical efficacy and safety data indicate that RP-L301 is a potential treatment for patients with severe PKD, including those who did not derive benefit from available therapies. Updated safety and efficacy including additional pediatric data will be presented.

OR06

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 (HSC) gene therapy allows the permanent reconstitution of the immune system with cells that have been genetically modified *ex vivo* by lentiviral transduction to express a specific therapeutic transgene. Upon proper myeloablative conditioning, cells derived from transplanted HSCs are also able to migrate and engraft, among other organs, in the brain, where they differentiate into microglia-like cells. These cells can then express locally in the central nervous system (CNS) the therapeutic transgene they have been transduced with. A powerful example of this is the prevention of severe neurologic defects in devastating diseases such as metachromatic leukodystrophy (MLD), where microglia replacement with gene modified HSC-derived microglia-like cells is a highly successful approach to deliver therapeutic molecules beyond the blood brain barrier (BBB) directly in the CNS, while this is unachievable by enzyme replace therapies (ERTs) due to the impermeability of the BBB itself.
Antibodies are effective therapeutic agents for a variety of pathologies. However, when delivered systemically, antibodies only minimally penetrate the BBB, and therefore they have very limited efficacy in the treatment of CNS disorders. Here, we sought to investigate whether *ex vivo* autologous HSC GT could represent an effective approach to deliver antibodies directly in the brain via engrafted gene-modified microglia-like cells.

First, we demonstrated that microglia cells can be gene modified *in vitro* to efficiently express and secrete a model single chain variable fragment (scFv) antibody, which then correctly binds to its target antigen when immobilised on a synthetic surface or expressed on the plasma membrane of HEK cells. Subsequently, we transduced HSCs with lentiviral particles carrying the scFv construct and optimised protocols to differentiate them into microglia cells *in vitro* to investigate the ability of HSC-derived microglia to efficiently secrete a functional antibody. The same HSCs were also transplanted into conditioned mice to analyse antibody production in the brain upon HSC engraftment and differentiation into microglia-like cells *in vivo*.

Microglia cells play a central role in neuroinflammation and they are particularly enriched in certain pathologies. The ability to harness their localisation for the target delivery of therapeutic antibodies could dramatically improve the prognosis of serious neurological conditions.

OR07

Acquisition of somatic mutations after hematopoietic stem cell gene therapy varies among cell lineages and is modulated by vector genotoxicity and the activity of key cellular senescence gene

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The hematopoietic system of patients undergoing Hematopoietic Stem and Progenitor Cell (HSPC) Gene Therapy (GT) is fully restored when autologous engineered HSPCs are reinfused into the patient. During this process, HSPCs go through a high level of proliferation until the hematopoietic reconstitution is complete. The impact of proliferation in HSPCs on cellular fitness and safety remains an open question. Moreover, the accumulation of somatic mutations in vivo could show differences in different hematopoietic lineages depending on their susceptibility to the negative effects elicited by the DNA damage response. Furthermore, oncogene activation in human HSPCs has been shown to trigger a chronic inflammatory response leading to hematopoietic decay.

Here we studied the clonality and the accumulation of somatic mutations in different hematopoietic lineages and during hematopoietic reconstitution in mice subjected to HSPC-GT. Indeed, wild type C57 mice were transplanted with bone marrow-derived lineage negative (Lin-) cells from WT mice or tumor-prone *Cdkn2a*$^{-/-}$ mice which lack p16^{INK4A} and p19^{ARF} proteins and thus have no barriers against proto-oncogene activation. Moreover, to evaluate if genotoxic integrations may increase the probability of acquiring somatic mutation upon oncogene activation, Lin- cells were transduced with a genotoxic LV harboring the strong retroviral enhancer/promoter Spleen Focus Forming Virus in the LTR (LV.SF.LTR) or the safer GT-like non-genotoxic LV (SIN.LV.PGK).

Mice receiving WT Lin- cells treated with LV.SF.LTR (N=25) or SIN.LV.PGK (N=24) did not develop tumors, while mice transplanted with *Cdkn2a*/LV.SF.LTR-marked cells (N=24) developed tumors significantly earlier compared to mock (N=20, $p<0.0001$) and mice receiving *Cdkn2a*/SIN.LV.PGK-treated cells (N=23, $p<0.0001$). To evaluate the clonal dynamics of hematopoietic reconstitution, vector integration sites (IS) were identified by by Sonication Mediated Integration Site (SLiM) PCR from peripheral blood, lymphoid (B and T) and myeloid cells collected every 4 weeks post-transplantation. Somatic mutations were identified by analyzing the

mouse genomic portion flanking each IS using VarScan2. Overall, we detected >200,000 IS, corresponding to more than 135 Mb of genomic sequence information. We introduced a new Mutation Index (MI), which normalizes the number of mutations by clones and coverage to assess mutation accumulation rates. By this approach, we found that the MI increased over time in LV.SF.LTR-treated mice and was significantly higher when compared to SIN.LV.PGK-treated mice ($p<0.001$). Notably, myeloid clones exhibited a higher frequency of mutation accumulation compared to T and B cell lineages. This phenomenon was further exacerbated in Cdkn2a/LV.SF.LTR-marked cells, indicating that the absence of barriers to proto-oncogene activation and the presence of genotoxic insertions result in progressive somatic mutation accumulation and insertional mutagenesis.

These results demonstrate for the first time that by combining the assessment of acquired mutations with IS analysis at the single clone level we can identify differential accumulations of somatic mutations in different hematopoietic lineages in vivo which depend on the genotoxic potential of the vector used and the ability of the genetically modified cells to sense and react to genotoxic lesions.

OR08

In vivo hematopoietic stem cell gene therapy using BaEVRLess-pseudotyped retroviral vectors

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In vivo hematopoietic stem cell (HSC) gene therapy has several potential advantages over *ex vivo* gene therapy, such as eliminating the need for stem cell harvest, *ex vivo* cell manipulation and conditioning of the patient. The VSVg envelope glycoprotein is commonly used for the pseudotyping of retroviral vectors but is not well suited for *in vivo* application due to its serum sensitivity and poor ability to mediate gene transfer into quiescent HSCs. In contrast, the baboon endogenous retrovirus (BaEV) glycoprotein, and its derivative BaEVRLess, mediate efficient gene transfer into resting HSCs and are serum resistant. Here, we explore the potential of BaEVRLess-pseudotyped retroviral particles for *in vivo* HSC gene therapy. Initially, to overcome problems during virus production related to high fusogenic activity of the BaEVRLess envelope, we generated a stable BaEVRLess-packaging cell line carrying knockout of the viral entry receptor ASCT2. This resulted in a 2-fold increase in titers (10^8-10^9 infectious particles/mL) and a 3-fold reduction in cytotoxicity. To reduce phagocytosis and immune responses *in vivo*, CD47 was overexpressed and beta-2-microglobulin was knocked out in the packaging cell line, respectively. Next, we tested BaEVRLess-pseudotyped lenti- and alpha-retroviral vectors in competitive *in vivo* gene transfer experiments, which revealed that both vectors transduce CD34+ cells at similar rates. To enhance gene transfer, we first optimized the mobilization of HSCs in humanized NBSGW mice. The combination of AMD3100 and Gro-beta efficiently mobilizes HSCs within 60 minutes of drug application in a range of 3-112 hCD34+ cells/µL of blood. 0.5-1 x 10^8 viral particles were

injected intravenously into animals after mobilization. Initial gene marking reached up to 1.9±0.9% of hCD45+ cells seven days post injection. To enrich gene-marked cells, we overexpressed MGMT-P140K in transduced cells, which mediates resistance to the alkylating agent BCNU. After three cycles of BCNU treatment, we observed a 20-fold (0.7% to 21.1% GFP+) or 144-fold (0.38 to 54.6% GFP+) enrichment of gene marked cells in the peripheral blood using either a low escalating or a high dose regimen, respectively. In bone marrow CD34+ cells, gene-marking averaged 18.1±9.1%. Barcode analysis revealed the presence of 300-640 unique barcodes and a polyclonal distribution in transplanted mice. To confirm transduction and selection of HSCs, secondary transplantations were performed, which revealed 39.3-48.1% transduced hCD45+ cells in the bone marrow of secondary recipients. In proof-of-concept experiments, we targeted the transcription factors BCL11A and ZNF410 using miRNA-embedded shRNAs (shmiR) containing vectors. Downregulation of BCL11A and ZNF410 in erythroid cells leads to sustained reactivation of gamma-globin and induction of fetal hemoglobin, which largely attenuates the hematologic effects of sickle cell disease. Following *in vivo* gene transfer and BCNU selection in humanized NBSGW mice, we observed a significant upregulation of gamma-globin (35.6%) and fetal hemoglobin (25.1%) in erythroid differentiated cells. In summary, we demonstrate the proof-of-principle that BaEVRLess-pseudotyped retroviral particles can be applied for *in vivo* gene therapy to treat sickle cell disease.

OR09

Zinc finger activators restore normal gene and protein expression in a mouse model of SCN2A haploinsufficiency

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SCN2A haploinsufficiency is caused by *de novo* loss-of-function mutations in the *SCN2A* gene resulting in insufficient gene expression and abnormally low levels of its encoded protein, neuronal voltage-gated sodium channel $Na_v1.2$. Patients with mutations causing *SCN2A* haploinsufficiency suffer developmental delays commonly associated with autism spectrum disorder (ASD) and intellectual disability (ID) for which no effective treatment exists. A potential therapeutic approach to treating genetic haploinsufficiency disorders involves transcriptional upregulation of the healthy allele to restore normal protein levels. We utilized our proprietary zinc finger transcriptional activator (ZF-A) platform to upregulate *Scn2a* expression *in vitro* and *in vivo* in wildtype (WT) and *Scn2a*$^{+/-}$ mice. Several hundred ZF-As targeting multiple transcription start sites were designed and screened to identify hot spots for gene activation. We identified several ZF-As targeting the *Scn2a* promoter that upregulated the target gene up to two-fold with high specificity. Selective upregulation of gene expression by ZF-As subsequently increased and restored $Na_v1.2$ protein to normal levels *in vitro* in both WT and *Scn2a*$^{+/-}$ mouse cortical neurons. These results translated *in vivo* in WT adult mice treated intravenously with AAV ZF-A whereby a 1.5-fold increase in *Scn2a* expression was confirmed in multiple brain regions using bulk tissue RT-qPCR. Upregulation of *Scn2a* levels was further confirmed at the single-cell level via multiplexed *in situ* hybridization and immunohistochemistry (ISH/IHC). Corroborating the results in WT mice, *Scn2a* upregulation was also observed in the brains of *Scn2a*$^{+/-}$ mice treated with AAV ZF-A four weeks following intracerebroventricular (ICV) administration. Taken together, our results demonstrate that targeted upregulation of *Scn2a* by ZF-As increases *Scn2a* expression *in vivo* and

restores $Na_v1.2$ levels to normal in *Scn2a*$^{+/-}$ mouse cortical neurons, thereby providing support for the advancement of a ZF-Activator approach to treat *SCN2A* haploinsufficiency.

OR10

Human RHO editing for treatment of autosomal dominant retinitis pigmentosa

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Retinitis Pigmentosa (RP) affects 1/3.000 patients worldwide, with 30-40% of cases having an autosomal dominant (AD) inheritance. The rhodopsin gene (RHO) is the most commonly mutated in AD RP patients (RP4), with the P23H gain-of-function mutation being the most common in US. Canonical gene therapy approaches that add a correct copy of the gene, are not beneficial and removing the mutant RHO allele is necessary to avoid its toxic effect. The CRISPR-Cas9 based allele-specific editing has emerged as a therapeutic option for dominant RP by blocking the production of the toxic protein without affecting the correct copy of the gene. However, this approach limits its applicability to all RP4 patients, as design of a gRNA specific for each mutation would be required. We have recently described Homology-Independent Targeted Integration (HITI) which uses adeno- associated viral (AAV) vectors to deliver both CRISPR-Cas9 and a donor template to be integrated at the mouse RHO locus. This blocks the expression of the endogenous RHO and replaces it with its wild type copy. Here we have designed AAV-HITI that targets the human RHO locus, and tested it in a P23H knock-in mouse model of RP4, wherein the endogenous RHO allele has been replaced by a red fluorescent protein tagged (RFP) human RHO harbouring the P23H mutation (hRHO-P23H-tagRFP).At 30 days post-injection, we have observed HITI efficiency up to 12±8% in the transduced area (n=4) and an improved retinal electrical function (B-wave) as measured by ERG (p=0,005; n=10). Prospectively, we plan to evaluate the improvement in the retinal phenotype at advanced timepoints and potential off-targets editing events. Our preliminary results provide the first proof-of-concept of the efficacy of HITI as a therapeutic strategy for AD RP due to RHO mutations in a humanised mouse model and therefore, it could be readily translated to a human setting.

OR11

Development and validation of a novel adeno-associated viral gene therapy for Mucopolysaccharidosis IIIB (MPSIIIB)

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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. Here we describe a novel AAV gene therapy using AAVPHP.eB-CAG-NaGlu vector. Following a unique intravenous and intraparenchymal administrations in MPSIIIB mice, high NaGlu activity was measured in the brain, cerebellum, and spinal cord starting 4 weeks post-treatment and persisted after 22 weeks post-treatment. This was accompanied by the normalization of GAG storage levels and improvements in lysosomal pathology and neuroinflammation. AAVPHP.eB-CAG-NaGlu treatment improved motor and cognitive functions. The vector biodistribustion and first assessment of tolerance combining intraparenchymal delivery in the white matter and intravenous delivery was performed in non-human primate. Post-surgery MRI images showed no oedema and perfect tolerance at 1 and 6 weeks post-surgery. A two-fold increase in NaGlu enzyme activity was measured in serum. NaGlu enzymatic activity in the different parts of the brain showed a very significant increase in the white matter, internal capsule, corpus callosum and in the caudate and putamen. Our data indicate that AAVPHP.eB-CAG-NaGlu is a promising treatment option for MPSIIIB patients.

OR12

Gene therapy for C9orf72-ALS reduces RNA toxicity and ameliorates behavioral phenotype in ALS mouse model

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The G4C2 repeat expansion in the chromosome 9 open reading frame 72 (*C9orf72*) gene is the most frequent mutation in familial amyotrophic lateral sclerosis (ALS). *C9orf72* mutation-harboring

transcripts cause accumulation of RNA foci and dipeptide repeat protein-mediated toxicity leading to neurodegeneration. Therapeutic microRNAs (miC9Os) were developed to decrease the pathologic transcripts. To provide proof of concept, AAV9-(G4C2)149 repeats-induced ALS mice were dosed intra-striatal with AAV-miC9Os. Expression of miC9Os was measured in the striatum and rostral cortex 18 weeks post-dosing. The most abundant miC9O variant was quantified by RT-qPCR and detected with fluorescent in-situ hybridization, confirming strong and widespread expression. Significant and dose-dependent knockdown of pathological *C9orf72* mRNA was achieved in the striatum and rostral cortex. Treatment with AAV-miC9Os led to a strong reduction of sense RNA foci and significant toxic dipeptide poly-glycine-proline lowering. Amelioration of the cognitive phenotype and improvement of muscle strength retention was observed in high dose AAV-miC9Os treated ALS mice. In addition, a non-human primate study was conducted with the lead AAV-miC9O using an intrathecal microcatheter to investigate tolerability and assess biodistribution using a clinically relevant route of administration.

OR13

Alpha-retrovirus-based virus-like particles for efficient CRISPR-Cas9 delivery into hematopoietic stem cells

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Electroporation of Cas9-RNP or mRNA is commonly used for Cas9-delivery into hematopoietic stem cells (HSCs). While efficient, these methods affect cell viability and physiology, require specific equipment, and are limited to *ex vivo* applications. Cas9-delivery by virus-like particles (VLP) is a promising alternative, such as the γ-retrovirus-based Nanoblade or eVLP systems. Here, we engineered alpha-retrovirus-based VLPs (RSV-VLP) for Cas9 delivery. We showed that these RSV-VLPs are 5-10 times more efficient than the current state-of-the-art eVLP system for Cas9 delivery using VSVg-pseudotyped particles as assessed by indel formation in various cell lines and human CD34+ HSCs. We identified optimal configurations through sequential testing of four different gag-Cas9 fusions, three viral protease sites and tuning the component ratios during VLP-production. Optimal editing was achieved by the fusion of Cas9 downstream of the p12 nucleocapsid coding region separated by the naturally present protease cleavage site. Significantly more Cas9 molecules per particle were present in RSV-VLPs compared to eVLPs (25±6 vs. 13±11 Cas9 molecules/particle), potentially explaining superior gene editing efficiencies. Over 90% indel rates were observed targeting multiple different loci with <1µL viral supernatant in cell lines ($1.1\text{-}9.8\times10^{8}$ editing viral particles/mL) and over 70% indel formation in HSCs using MOI 100 while maintaining viability equivalent to untransduced cells (95% viable hCD34+ cells). We additionally explored RSV-VLPs for multiplex editing, simultaneous delivery of an integrating payload, and flexible pseudotyping for editing quiescent HSCs during short *ex vivo* culture. Proof-of-concept studies targeting the therapeutically relevant *BCL11A* erythroid enhancer in HSCs resulted in 61.7±5.5% indel formation, which was associated with 52.1±5.7% gamma-globin expression and 18.6±0.3% fetal hemoglobin induction following erythroid differentiation. In summary, RSV-VLPs are an efficient and versatile tool for the delivery of CRISPR-Cas9 and have potential use for *in vivo* gene editing applications.

OR14

Programmable deaminase-free base editors for G and T base editing by engineered glycosylase

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Base editing is a novel evolution of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas based technology installing point mutations directly into the cellular DNA or RNA without inducing a double-stranded DNA break (DSB). Current base editors mainly contain programmable DNA-binding proteins, such as a catalytically impaired CRISPR-associated (Cas) nuclease that was fused with a single-stranded DNA deaminase enzyme. Base editors directly convert one base or base pair into another, enabling the efficient installation of point mutations in non-dividing cells without generating excess of undesired editing byproducts. However, all of these base editing methods begin with deamination of C or A as the key step to produce deoxyuridine (U) or deoxyinosine (I) intermediates, respectively, which in turn transforms into other bases by endogenous DNA repair or replication mechanisms. However, there is no available method for guanine (G) or thymine (T) editing since deamination of G rarely causes base conversion while T lacks amino groups. Here, we developed a deaminase-free glycosylase-based guanine base editor (gGBE) with G editing ability by fusing Cas9 nickase with engineered N-methylpurine DNA glycosylase protein (MPG). After several rounds of MPG mutagenesis via unbiased and rational screening using an intron-split enhanced green fluorescent protein (EGFP) reporter through our HG-PRECISE® (**H**uida**G**ene – **P**latform for **R**ational **E**ngineering **C**RISPR-Cas **I**dentification by **S**ynergic **E**xpertise) platform, we demonstrated the increase of G editing efficiency by more than 1,500 folds with our MPG-engineered gGBE. Furthermore, our novel gGBE exhibited up to 81.2% of base editing efficiency and up to 0.95 of G-to-T or G-to-C (i.e., G-to-Y) conversion ratio in both cultured human cells and mouse embryos. Furthermore, gGBE exhibited efficient G-to-Y conversion when replaced nCas9(D10A) with dCas9, Cas12, or IscB variants. Recently, we further improved the efficiency and purity of G-to-T editing outcomes and developed an engineering gGTBE, a more accurate gGTBE which may enhance the therapeutic potential of the base editor platform. Additionally, we also developed a deaminase-free glycosylase-based thymine base editor (gTBE) with T editing ability. Taken all together, we demonstrated that DNA glycosylases could be engineered into new types of base-editing tools by endowing them the capability to selectively excising different substrates.

OR15

MILESTONE, A universal CRISPR/Cas9n-mediated genome editing strategy for *ELANE-related* severe congenital neutropenia

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Severe congenital neutropenias (CN) are a heterogeneous group of rare hematological diseases that are characterized by impaired maturation of neutrophil granulocytes.
Autosomal-dominant *ELANE* mutations are the most common cause of 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. Although most patients respond to daily treatment with recombinant human granulocyte colony-stimulating factor, a group of patients does not respond to this cytokine, and approximately 20% of patients develop myelodysplasia or acute myeloid leukemia. Hematopoietic stem cell transplantation is the only curative therapy so far, but it is associated with severe adverse events. Gene editing of patients` HSPCs *ex vivo*, followed by autologous transplantation, offers a novel curative therapy. Due to the wide range of reported mutations that are distributed over all 5 exons of the *ELANE* gene, a universal, well-investigated, high-safety-profile strategy could benefit all *ELANE*-CN patients. Our group has already reported a universal strategy for *ELANE*-CN by CRISPR/Cas9-mediated *ELANE* knockout, but gene editing in the coding sequence region (CDS) of the *ELANE* gene might be potentially unsafe as any unwanted on-target event could be considered a new disease-causing variant. We aimed to reformat our previously reported knockout concept into a clinical-grade gene therapy approach with the highest level of safety.

We have screened *ELANE* core promoter and, using CRISPR/Cas9n, introduced two nicks on opposing strands of the Goldberg-Hogness box region (TATA-box) to make the transcription starting process ineffective. We termed the approach *MILESTONE* (**M**odifying *E**L**ANE* Goldb**e**rg-Hogne**s**s box **to** i**n**hibit **e**xpression). *MILESTONE* targets the non-coding region of the *ELANE* gene and thus does not generate new unwanted variants. Also, by replacing Cas9 with Cas9-ncikase, the off-target activity should decrease by up to 1000-fold.

Using the *MILESTONE* strategy, no disruption of granulopoiesis in healthy donor HSPCs (n = 2) was observed, while there was a markedly elevated neutrophil differentiation in gene-edited *ELANE*-CN patient's HSPCs (n = 2), as assessed by the percentage of $CD45^+CD11b^+CD15^+$, $CD45^+CD15^+CD16^+$, and $CD45^+CD16^+CD66b^+$ myeloid/granulocytic cells and compared to the mock electroporated *ELANE*-CN group. Similar results were achieved by the CFU assay. To have a nucleotide-level resolution of on-target events, we performed rhAMP-seq on gene-edited healthy donors or *ELANE*-CN HSPCs. Next-generation sequencing results showed >90% on-target efficiency.

Strikingly, *MILESTONE* restored defective granulopoiesis *in vivo*, as gene-edited *ELANE*-CN HSPCs (n = 3) were transplanted in NSG mice and assessed by the percentage of neutrophils ($hCD19^{-}hCD3^{-}hCD66b^{int/low}hCD33^{+}hCD16^{high}$) after 16 weeks.

MILESTONE safety investigations in healthy donor $CD34^{+}$ HSPCs depicted a safe profile as GUIDE-seq highlighted two potential off-target sites after >=6 mismatches, and CAST-seq detected no chromosomal translocations. Transcriptomic profiling of *in vitro* generated neutrophils confirmed a reduction of *ELANE* gene expression in gene-edited cells of up to 9-fold. At the same time, none of the other serine proteases or neighbor genes were down-regulated.

Taken together, *ex vivo* CRISPR-Cas9n-based gene editing of the *ELANE* gene using *MILESTONE* in the setting of autologous stem cell transplantation could be a safe therapeutic approach for all *ELANE*-CN patients.

OR16

DNA/RNA hybrids as double edge sword: r-loops can influence the efficiency of AAV-mediated genome editing and undesired vector integrations in vivo

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AAV vectors possess a natural ability to stimulate homologous recombination (AAV-HR). They were also reported to randomly integrate into numerous locations throughout the genome. We have demonstrated that a promoterless AAV vector containing host genomic homology arms, flanking a therapeutic coding sequence, induced homologous recombination in mouse liver. While this approach has led to therapeutic levels of a handful of proteins, the percentage of edited cells remains low (~ 1.0%).

To improve the efficiency of AAV-HR, a genetic screen identified that the inhibition of the Fanconi Anemia complementation group M (FANCM) significantly enhances the AAV-HR. FANCM is one of the enzymes required to resolve DNA/RNA hybrids, also known as r-loops, that can occur when a RNA filament anneals to a single strand DNA. R-loops are forming mainly during DNA transcription, and if not tightly regulated could trigger the cellular DNA damage response. However, r-loops can be physiologically resolved by specific helicases or enzymes belonging to the RNase H family which specifically degrade the RNA strand.

Here, we report that the genetic inhibition of two enzymes involved in r-loop formation results in a significant improvement of AAV-HR in vitro. In addition, the small molecule topotecan resulted in a significant increase in genome-wide r-loop levels in a murine hepatoma cell line (HEPA1-6) and mouse liver. Topotecan treatment resulted in a considerable enhancement of AAV-HR in vitro and in vivo. We were able to show the mechanism of improved AAV-HR efficiency was indeed due to r-loop formation by blocking the enhancement by co-treatment with RNAseH1.

We next elected to map and quantify r-loops throughout the genome. To this end, we performed DNA/RNA immune precipitation sequencing (DRIP-seq) studies in HEPA1-6 and liver. To our knowledge, this represents the first genomic r-loop map of an intact tissue. Notably, we were able to detect massive r-loops accumulation in the region of the *Albumin* locus (exons 12-14) where we had designed homology targeting arms (Barzel et al. Nature 2015). Conversely, when we targeted a

different *Albumin* region with very low r-loops levels, we did not observe any detectable vector-integration.

In addition, we also have preliminary data suggesting that regions enriched for r-loops are possible hotspots for “random” vector integration. For instance, we found a gene which shows a strong correlation of high r-loops levels and off-target AAV integration.

We are currently conducting bioinformatic analyses in order to identify prospective genomic features which might further explain the interplay between AAV integration and r-loops.

These findings may shed light on mechanisms for improving the safety and efficacy of genome editing and may enhance our ability to predict regions most susceptible to insertional mutagenesis with canonical AAV vectors.

OR17

In Vivo targeting of HSC by capsid-engineered AAV vectors

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In vivo targeting of hematopoietic stem cells (HSC) possesses the potential to substantially empower cell therapy-based strategies and to broaden the applicability of gene therapy-based strategies. We aim to reach this goal by tailoring the adeno-associated viral (AAV) vector system. Specifically, we performed a high-throughput AAV peptide display library screen for capsid variants targeting hematopoietic stem and progenitor cells (HSPC, CD34+) in non-human primates (NHP). We designated a set of highly promising capsids recovered from the isolated NHP CD34+ cells for further investigation as barcoded (BC) eGFP vectors in CD34+ humanized NSG mice (i.v., n= 6-7/pool, 5 d). While FACS analysis revealed moderate GFP signals in PBMCs and bone marrow (BM)-derived CD34+ cells arguing for successful in vivo transduction following intravenous administration, NGS analysis of mRNA BC enabled the identification of the capsids with a dominant contribution to the mRNA expression in hCD34+ cells (% of total BC reads: 20-60 %). Furthermore, *ex vivo* transduction experiments identified a set of variants with superior *ex vivo* hCD34+ cell transduction efficiency (eGFP+ cells 60-85 %). For a more detailed investigation of the improved capsid tropism, the uncoating efficiency of the above-mentioned CD34+ cell-tropic capsids was compared in a BC.eGFP vector pool administered to hCD34+ NSG mice (i.v., n= 6-7, 5 d), with AAV6 and AAV2 as controls. Here, encapsidated vs. uncoated vector genomes (vg) were analyzed by NGS. In detail, genomic DNA derived from CD34+ cells, PBMC, and liver was T5 exonuclease treated to deplete linear vector DNA (encapsidated) and rescue circular concatemeric vector DNA (uncoated). Upon observation of substantial differences between the capsids, two variants, Var24 and Var30, in comparison to AAV2 and AAV6, were subjected to an NHP BC.eGFP AAV study on efficient CD34+-targeting (i.v., n= 1, 7 d). Strikingly, NGS analysis of the BC AAV mRNA distribution revealed the superior time-dependently increasing contribution of Var30 to CD34+ mRNA BC expression (% of total BC reads: 50 % (3 d); 90 % (7 d)). Moreover, Var24 mediated highly selective transgene expression in CD90+ long-term HSPC (% of total BC reads: 32 % (7 d)) in contrast to neglectable Var24 BC contribution to liver mRNA BC expression (% of total BC reads: 1 % (7 d)). NGS studies on BC distribution among uncoated vg are ongoing. In conclusion,

we successfully performed a CD34+ cell-targeted AAV2 capsid variant library selection in an NHP model and confirmed the species-independent tropism of several promising capsids for CD34+ cells *ex vivo*, in humanized NSG mice, and NHP. In particular, Var30, with its highly efficient *in vivo* tropism for CD34+ cells, and Var24, with its high selectivity for long-term HSPC, represent strong candidates to equip vectors for gene therapeutic *in vivo* delivery to HSC.

OR18

An integrin-targeting AAV developed by a novel computational rational design methodology presents an improved targeting to the skeletal muscle and reduced tropism to the liver

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Adeno-associated virus (AAV) has shown potential as a gene therapy vector due to its efficiency in delivering transgenes and low toxicity. However, its effectiveness can be limited by non-optimal tissue targeting and accumulation in the liver. High doses are often required to achieve therapeutic efficacy, particularly in the treatment of muscular diseases where the target tissue represents half the body mass. Such doses can lead to severe adverse effects, including hepatotoxicity as seen in recent clinical trials. With the primary aim to lower treatment doses, we present here a novel methodology for AAV computational rational design targeting skeletal muscle, resulting in a new myotropic AAV variant. First, integrin alpha V beta 6 (AVB6) was selected from screening of potential receptors with high enrichment in skeletal muscle tissue compared to other organs. A liver-detargeting AAV capsid backbone, a hybrid between AAV-9 and -rh74 (AAV9rh74), was engineered to bind specifically to AVB6. Inspired by recent successes of various protein design studies, we modified the entire sequence and structure of variable region IV loop from its predicted 3D structure, in such a way that the designed capsids acquire the RGDLxxL/I motif structure with low estimated energy. It allows a high-affinity binding to AVB6 but remains stable, thus better AAV production. Five AAV variants from *in silico* design were experimentally tested. Compared to AAV9rh74, all designed AAVs showed better productivity, better binding to AVB6, and greater infectivity towards human differentiated myotubes and murine skeletal muscles *in vivo* while entering poorly in the liver. One notable variant, LICA1, greatly increases transgene expression at 16.6/25.0-fold greater in human myotubes, and murine skeletal muscle at 13.7/129.3-fold greater than AAV9 and AAV9rh74, respectively. We further examined its efficacy, in comparison with AAV9, in delivering therapeutic transgenes in mouse models of Duchenne muscular dystrophy and limb-girdle muscular dystrophy R3 at a low dose of 5E12 vg/kg. At this dose, AAV9 is suboptimal where it can only infect 22.1-58.1% of total muscle fibers. In contrast, LICA1 effectively delivered and expressed transgenes in 74.8% myofibers in severely affected diaphragm, or at almost 100% in other tested muscles. Consequently, LICA1 corrected pathohistology by lowering centro-nucleation index and fibrosis, and restored global transcriptomic dysregulation. Compared to AAV9, the anti-capsid antibody in LICA1 one-month treatment in DMD mice halves and lower titers of neutralizing antibodies were observed in a small human serum cohort indicating a lower immunogenic profile. Furthermore, compared to other published state-of-the-art myotropic AAVs, LICA1 is better at production, and targets skeletal more specifically since accumulates significantly less in the liver. These results suggest the potential of our design method for AAV engineering and demonstrated the interest of the novel AAV variant for the gene therapy treatment of muscular diseases.

OR19

DART-AAVs enable specific transduction of murine and human CD8 T cells for *in vivo* gene therapy

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One of the biggest challenges of many *in vivo* gene therapy applications, including *in vivo* CAR-T cell therapy, is the lack of vector systems for highly specific gene transfer selectively into a defined population of therapy-relevant cells. 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 present DARPin-targeted AAVs (DART-AAVs), a second-generation receptor-targeted AAV, exhibiting improved DARPin display and gene transfer activity. By inserting a murine CD8-specific DARPin into the GH2-GH3 loop of the AAV2 viral capsid protein 1 (VP1) we not only achieved >99% gene transfer selectivity on CD8+ murine splenocytes, but also higher gene transfer rates than those mediated by unmodified AAV2. Additionally, when systemically injected into a syngeneic Ai9 reporter mouse model, mCD8-AAV having the Cre recombinase gene packaged exhibited a more than 20-fold reduced liver burden as compared to AAV2. Flow cytometry analysis revealed highly selective transduction of CD8+ cells in spleen, blood and bone marrow (90.1/99.2/87.4 percentage of CD8+ among tdT+ cells) demonstrating precise delivery of the recombinase into CD8+ cells by mCD8-AAV. Based on these promising results, the human CD8-specific DARPin 63A4 was inserted into the GH2/3 loop of AAV2 VP1. Vector stocks of hCD8-AAV having a GFP reporter packaged were indistinguishable from unmodified AAV2 stocks in terms of titers and particle morphology. High-resolution cryo-electron microscopy confirmed an unaltered particle structure. When incubated with CD8+ SupT1 cells 86 ± 3 % of all cells expressed the reporter at a GOI of 2.5×10^5. Notably, off-target transduction on CD8-negative A301 cells was close to background with 0.8 ± 0.1 % GFP+ cells even at a GOI of 6.67×10^5. When incubated with blood cells from human donors (GOI of 5×10^5), hCD8-AVV bound 88 ± 8 % (n = 2) of all CD8+ cells but not to CD8-negative 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.

These data demonstrate that capsid insertion of DARPins mediates highly selective and efficient gene transfer into mouse and human T lymphocytes. Thus, DART-AAVs have the potential to broaden *in vivo* gene therapy options substantially and to facilitate translatability between animal preclinical studies and human applications.

OR20

Directed evolution of novel AAV-capsid variants for efficient and specific targeting of primary human Schwann cells

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Therapeutic strategies aiming at delivering functional copies of mutated non-functional genes (gene augmentation) or editing disease-causing mutations (gene editing) hold great promise for treating many devastating genetic diseases. Despite recent clinical successes, safe and efficient delivery of therapeutic genetic modifiers to specific cells *in vivo*, especially in the peripheral nervous system, remains challenging. Recombinant vectors based on adeno-associated virus (AAV) have become the delivery system of choice for *in vivo* applications. However, many of the currently utilised AAV variants are based on natural serotypes that evolved for optimal host infection and not for clinical applications.

Schwann cells (SC), crucial for peripheral nerve protection and regeneration, are therapeutic targets for various conditions, including neurofibromatosis and spinal cord injuries. Unfortunately, currently, available AAV variants cannot efficiently target these cells. Therefore, Schwann cell-tropic AAVs are required to enable the development and clinical implementation of novel promising SC-targeted gene therapies.

In this study, we used our proprietary functional transduction (FT) selection platform to perform the first-ever AAV-directed evolution on primary human Schwann cells. The FT platform enables high-throughput identification of novel AAV variants based on transgene expression, not just cellular entry. Thus, we hypothesised that the application of the FT platform would support the selection of the most functional AAV variants for clinical gene augmentation applications targeting hSCs.

Using the FT platform, we performed AAV library selection in a cross-system strategy involving *in vitro* and *in vivo* models of hSCs. Specifically, the *in vitro* selection was done on primary hSCs and two unique variants were identified, named Pep2hSC1 and Pep2hSC2. Both variants exhibited improved SCs transduction with higher specificity compared to AAV-DJ, the most functional variant for SC targeting reported to date. We confirmed the hSCs transduction efficiency of Pep2hSC1 and Pep2hSC2 in a direct comparison using hSCs derived from primary nerve and skin from seven human donors of different ages and sexes. Importantly, from the perspective of *in vivo* therapeutic application, our variants showed higher specificity for hSCs in mixed SCs and fibroblast cultures. Specifically, Pep2hSC1 transduced fibroblasts at a level similar to AAV-7m8 (control) and significantly less efficiently than AAV-DJ. In comparison, Pep2hSC2 did not transduce human fibroblasts, positioning this variant as a strong candidate for *in vivo* gene therapy applications. As the liver is a major off-target organ for all AAVs, to ensure maximum safety in clinical applications, we used a xenograft mouse model of the human liver to evaluate the new variants for their ability to transduce primary human hepatocytes *in vivo*. Both variants exhibit lower transduction of human hepatocytes (at the DNA level) and lower transgene expression (at the RNA/cDNA level) compared with AAV-DJ.

In conclusion, our novel Schwann Cell tropic AAV capsid variants, Pep2hSC1 and Pep2hSC2, show improved specificity and transduction efficiency of primary human Schwann Cells and reduced tropism towards human primary hepatocytes *in vivo*. Based on our data, these novel vectors may have the potential to address current limitations of AAV-based therapies targeting SCs and serve as a basis for developing next-generation Schwann Cell-tropic capsids.

OR21

Rescue of a lethal murine model of methylmalonic acidemia after AAV delivery of liver specific and global piggybac transposase systems

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Methylmalonic acidemia (MMA) is a severe autosomal recessive metabolic disorder caused most frequently by variants in the methylmalonyl-CoA mutase (*MMUT*) gene, which encodes for this mitochondrial localized enzyme. MMA results in both acute and chronic multisystemic medical complications that can be fatal. Severely effect patients can undergo liver transplantation to treat the underlying enzymatic defect and the success of this procedure suggests that liver direct gene delivery maybe a viable treatment option, but the multisystemic nature of this disease suggest that correction of both hepatic and non-hepatic tissue could be beneficial. Systemic AAV gene therapy has recently emerged as a promising new therapy for MMA but could be limited by the temporal loss of AAV episomes, a well-recognized limitation of therapy with non-integrating vectors. Here we explore the therapeutic efficacy of both a liver specific and global transposase system to integrate a therapeutic transgene into the cells of mice using a recently generated mouse model of a lethal form of MMA caused by a MMUT p.R106C variant. Therapeutic AAV transgenes were created for each transposase and cloned between AAV2-ITRs configured with transposon-specific terminal repeats (TR): one AAV expresses a *MMUT* transgene under the control of a liver specific promoter and the other, a constitutive promoter. A second set of AAV vectors were designed to express the Piggybac transposases in a liver specific or constitutive manner. When both AAVs transduce the same cell, the transposase mediates a cut-and-paste insertion of the *MMUT* transgene into the genome. Either the transgene only or transgene plus transposase vectors were delivered at birth by retroorbital injection, at doses of 1e13 to 5e13 vg/kg per vector, to $Mmut^{R106C/R106C}$ mice and control littermates. $Mmut^{R106C/R106C}$ mice treated with either the liver specific or constitutive transposase system and MMUT transgenes had significantly increased survival, improved growth and reduced plasma methylmalonic acid versus both untreated and transgene only treated mice. Plasma methylmalonic acid levels were significantly lower than in untreated mice at all time points measured out to 8 months posttreatment ($p<0.01$). Lastly, in situ hybridization to detect transgene expression in the livers harvested from mice treated with the liver specific transposase system revealed large numbers of hepatocytes expressing *MMUT* mRNA at one- and 6-months posttreatment, with abundant positive staining hepatocytes, widely distributed throughout the liver. AAV delivery of these transposase vectors enabled integration of a therapeutic transgene and the permanent correction of the clinical manifestations of MMA in a disease model with a severe phenotype. The modular design of the vectors we have developed should enable the rapid assessment of other transposable elements for therapeutic benefits in the treatment of MMA, and by extension, other disorders of intermediary metabolism where early hepatic or global tissue correction is beneficial, and stable transgene expression is required.

OR22

A single, systemic administration of BEAM-301 mitigates fasting hypoglycemia one year after dosing in a transgenic mouse model of glycogen storage disease type Ia

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Glycogen storage disease type Ia (GSD-Ia) is an inborn error of metabolism caused by mutations in the *G6PC* gene that inactivate glucose-6-phosphatase-α (G6Pase-α or G6PC), a main enzyme in regulating interprandial euglycemia. GSD-Ia patients exhibit impaired glucose homeostasis and life-threatening fasting hypoglycemia, in addition to accumulation of hepatic glycogen and lipids, resulting in enlarged liver. The active site mutation, *G6PC*-p.R83C, is a common and severe pathogenic mutation identified in Caucasian GSD-Ia patients and contains a single G-to-A transition mutation in the *G6PC* gene. Adenine base editors (ABEs) enable the programmable conversion of A•T to G•C in genomic DNA. Here, we evaluated the long-term efficacy of a novel ABE to correct the *G6PC*-p.R83C mutation in a transgenic mouse model of GSD-Ia, homozygous for human *G6PC*-p.R83C (huR83C) and deficient of G6Pase-α activity. Given the high rate of neonatal lethality in huR83C mice, subjects were dosed shortly after birth with BEAM-301, a lipid nanoparticle (LNP) containing guide RNA and mRNA encoding ABE. BEAM-301-dosed huR83C mice grew normally to at least 1 year of age and exhibited base-editing efficiencies of up to ~60% in total liver extracts. Remarkably, just single digit percentage base-editing rates were sufficient to restore physiologically relevant levels of hepatic G6Pase-α activity, normalize serum metabolites, and most importantly prevent hypoglycemia during successive 24h fast challenges within 1 year (at 20wks, 35wks, and 52wks of age). Furthermore, BEAM-301-dosed animals exhibited near-normal whole liver size, with reduction in both lipid accumulation and hepatocyte size confirmed via histology. In summary, these data demonstrate durable and clinically-relevant correction of *G6PC*-p.R83C and further support the development of BEAM-301 as a potential one-time treatment for GSD-Ia.

OR23

Lentiviral-based liver gene therapy provides long-term efficacy and safety, global restoration of liver pathology and therapeutic benefit in kidney and brain, in a mouse model of methylmalonic acidemia

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Methylmalonic acidemia is a severe metabolic disease caused by deficiency of the mitochondrial enzyme methylmalonyl-coA mutase (MUT). The resulting build-up of toxic methylmalonic acid (MMA) leads to multisystemic life-threatening complications including chronic renal disease and neurological disabilities. The significant MMA-related morbidity and mortality in infancy led to liver transplantation as elective treatment to stabilize these patients. Integrative lentiviral vector (LV)-based gene therapy may represent an alternative to liver transplantation, allowing for a stable gene transfer even in young children at initial disease stage. Here, we assessed the long-term efficacy and safety of a liver-directed LV gene therapy in an intermediate mouse model (MCK-Mut-/-) of the disease. We assessed the disease progression in this mouse model by measuring MMA levels in liver and kidney, which were significantly higher compared to those of age-matched wild-type littermates already at 2 weeks of age. The MMA accumulation was even more pronounced in 6 month and 12 month-old animals, thus indicating a progressive worsening of the disease. We employed a LV encoding a human MUT transgene under the control of a hepatocyte-specific expression cassette (LV.MUT). We administered 2 week-old MCK-Mut-/- mice and monitored them for >1 year. Treated animals showed rapid, substantial and long-lasting decrease in circulating MMA (mean of 146 μM and 979 μM in treated vs. untreated animals 12 months post-LV) with normalization of plasma FGF-21, a key biomarker of the mitochondrial function. RNA in situ hybridization analysis showed up to 20% of liver transduced area. Liver overexpression of MUT enzyme per cell, estimated at 8-10 fold over the endogenous expression by mRNA analysis, resulted in normalization of the mitochondrial shape and structure, as assessed by electron microscopy. The liver histology at the end of the experiment was fully normal in treated mice, which were comparable to wild-type controls and did not display the parenchymal alterations found in MCK-Mut -/- untreated mice. In line with these results, the concentration of intrahepatic MMA was almost completely normalized (mean of 0.3 μM, 5.3 μM and 300 μM in wild-type, treated and untreated animals respectively), suggesting detoxification activity of the corrected hepatocytes over the non-corrected ones. Importantly, we observed significant MMA reduction also in the brain (28 μM and 93 μM in treated vs. untreated animals) and in the kidney (81 μM and 552 μM in treated vs. untreated animals) with correction of the mitochondrial alterations, indicating an extrahepatic therapeutic benefit achieved by liver detoxification. We treated additional MCK-Mut-/- mice at different LV doses (2.5, 5 or $10x10^{10}$ TU/kg) and we observed a dose-dependent correction of the disease biomarkers. Mice treated at the lower LV dose displayed a progressive rescue, with full weight normalization and a decrease in circulating MMA comparable to those of mice injected at higher LV doses, only around 3 months post-LV. This kinetics suggest a selective advantage of the corrected cells, especially when starting from an initial lower LV dose/liver transduction. Overall, these data provide evidence for the efficacy, safety and extrahepatic benefit of liver mediated LV gene therapy for methylmalonic acidemia.

OR24

Prenatal in vivo base editing for the treatment of Krabbe disease

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Infantile Krabbe disease (KD) is an autosomal recessive genetic disorder characterized by a severe and progressive neurodegeneration leading to fatal outcome within the first 3 years of life, caused by mutations in gene encoding for the lysosomal hydrolase galactocerebrosidase (GALC). Clinical manifestations are neurological, due to de-myelination in the central and peripheral nervous systems (CNS and PNS), astrocytic gliosis, and neuroinflammation. Currently, KD remains without a definitive treatment. Hematopoietic stem cell transplantation can delay the onset and progression of the disease thanks to GALC reconstitution in CNS tissues by hematopoietic stem cells-derived myeloid cells and only when performed in pre-symptomatic newborns, highlighting the narrow therapeutic opportunity window. Therefore, an effective and safe therapy that can correct the underlying mutations, prevent early-onset neuropathy, and preserve the developing brain is urgently needed. In this regard, we are developing a prenatal, in-vivo gene editing platform to mediate *Galc* mutations correction in the CNS, PNS and hematopoietic system using adenine base editors (ABEs). *In utero* intervention brings many advantages, including early treatment before disease onset, increased accessibility to the brain and to proliferating cell progenitors, high immune tolerance and reduced BE doses needed. We have generated engineered Virus-Like Particles (eVLPs) containing the ABE8e-NG and a cognate guide-RNA targeting the non-sense point mutation (TG**A**>TG**G**) that introduces a premature stop codon in the *Galc* gene, thus leading to absence of protein expression and the onset of the disease in the KD Twitcher mouse model. The use of ABE to correct point mutations in situ guarantees a permanent therapeutic benefit compared to current strategies based on Adeno-Associated Vector-delivered GALC cDNAs and, when packaged into eVLPs, it ensures a fast, transient, and widespread delivery to many cell types involved in KD. For the initial proof-of-concept, we have developed a reporter system harbouring a portion of the mutated *Galc* gene fused to an mCherry reporter gene, where ABE-mediated correction of the *Galc* mutation activates mCherry expression. Using this reporter system in combination with ABE-eVLPs in 293T cells, we achieved up to 95% correction frequency of the target mutation as assessed by mCherry protein expression by flow cytometry, immunoblotting and enzyme linked immunosorbent assay (ELISA). We then tested the ABE-eVLPs in an immortalized KD Schwann cell line by transduction in a dose dependent manner, to find the best dose capable of effective base editing without compromising cell viability. Analysis of GALC protein reconstitution showed a 2-3-fold increased gene expression in edited KD Schwann cells as compared to unmanipulated controls. We are now progressing to *in vivo* testing of our BE-eVLP platform by their *in utero* injection in Twitcher pregnant female mice to assess functional gene correction and GALC protein expression, as well as biodistribution of eVLPs in the foetal and surrounding maternal tissues. Our final aim is to develop a safe and efficient *in vivo* BE platform to treat KD, which could ideally serve as a proof-of-concept therapeutic platform for other early-onset lysosomal storage disorders and neurodegenerative diseases.

OR25

Engineering AAV variants via rational design and directed evolution to escape pre-existing vector immunity

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A major clinical obstacle to the application of vectors derived from adeno-associated virus (AAV) is pre-existing humoral immunity to the viral capsid induced by natural AAV infection. Additionally, vector-induced immunity will also preclude patient re-treatment should there be an inadequate initial therapeutic response, or if the therapeutic benefits start to wane. Strategies under development to overcome pre-existing immunity can be broadly divided into those that seek to alter the host response to the vector or those that seek to modify the vector itself. The current project seeks the latter by modifying the AAV9 capsid through parallel yet cross-complementary pathways: (i) structure-guided capsid engineering to 'erase' antibody epitopes and (ii) Darwinian selection of antibody-escape variants. We have recently reached a significant milestone in achieving this goal by isolating a panel of human anti-AAV9 monoclonal antibodies (mAbs, n=35) and functionally and structurally characterising a subset (n=21). The generation of this valuable resource was facilitated by access to samples collected from three patients who had received the AAV9 gene therapy Zolgensma to treat spinal muscular atrophy. The treated infants possessed a high frequency of anti-AAV9 reactive switched memory B cells (smB cells) from which the mAbs were cloned. Many of the anti-AAV9 mAbs showed binding affinities in the sub-nanomolar range (11/21 mAbs) and all neutralised AAV9 entry with 4 mAbs cross-neutralising other AAV capsid serotypes. Cryo-EM revealed four distinct capsid binding patterns, akin to those described for mouse-derived mAbs, with the majority of human mAbs (16/21mAbs) binding the 2-fold axis of symmetry in contrast to murine mAbs, which bind predominantly at the 3-fold axis. Cryo-EM analysis at 2-3 Å resolution is revealing the molecular structures underlying the mAb-capsid interactions and permitting the rational design of AAV9 variants that evade binding of 15/21 mAbs. Additionally, we have also developed a novel high-throughput Darwinian-selection model that applies the mAb against AAV9 capsid libraries PCR-generated to contain all single amino acid changes at each capsid residue. The model not only permits the exploration of all single amino acid capsid changes on vector escape from mAb neutralisation, but simultaneously quantifies the relative enrichment for each change that permits escape. Cross-correlation of the Darwinian-selection model against the structure-guided approach has been undertaken using a mAb for which a variant has already been engineered by rational design. As predicted the model detected single amino substitutions at Q588 thereby validating the performance of the model. Our team are now turning attention to identify capsid variants for mAbs where rational design has been unsuccessful. Collectively, this unique pipeline provides valuable opportunities for both basic scientific discovery and AAV vector engineering to ultimately enable universal access to transformative AAV gene therapies.

OR26

Dose-dependent inflammation signatures following Ixo-vec administration in non-human primates

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Wet age-related macular degeneration (wAMD) is a retinal disease mediated by the abnormal production of vascular endothelial growth factor (VEGF), where the growth of abnormal, leaky blood vessels in the macula leads to a reduction in central vision. The anti-VEGF drug aflibercept can reverse the leakage of fluid and dry the retina but requires frequent bolus injections, which represent a substantial treatment burden to patients and the health care system. Ixo-vec is a recombinant adeno-associated virus (rAAV) that harnesses ocular cells to become biofactories that express and secrete aflibercept. Dose dependent mild to moderate inflammation that was responsive to topical corticosteroid therapy was seen at 2E11 and 6E11vg/eye doses in the OPTIC study. Phase 2 LUNA study is evaluating 2E11 vg/eye and a new, lower 6E10 vg/eye dose to expand the known therapeutic window. Findings from nonhuman primate (NHP) studies can help optimize the risk benefit profile in humans. NHP data has demonstrated intravitreal (IVT) administration of Ixo-vec resulted in a non-dose proportional aflibercept levels across several orders of magnitude of Ixo-vec dosing. Levels at 3E10 vg/eye (human equivalent dose [HED] 6E10 vg/eye) were nearly equivalent to aflibercept levels observed at higher doses (up to 2E13 vg/eye). NHP data has also demonstrated dose-dependent inflammation, with little to no inflammation at the 3E10 and 1E11 vg/eye doses to inflammation requiring corticosteroids at 2E12 vg/eye (HED 4E12 vg/eye) or greater. We assessed potential signatures of inflammation including toxicity related to overproduction of exogenous protein and general pathways previously identified in gene therapy studies. Mechanisms were tested by systems-based transcriptomic analysis. Three months following IVT administration of Ixo-vec the choroid, retina, and anterior region tissues of NHPs were isolated for bulk RNA-seq. Pathway analysis was performed using three different software tools. The outcomes of these different analyses were highly convergent in identifying the same pathways of interest as dysregulated in a dose-dependent manner. Unfolded protein and endoplasmic reticulum stress responses were not upregulated, indicating that toxicity due to overproduction of exogenous aflibercept did not cause inflammation at higher doses. Furthermore, there was no evidence of disrupted ciliary body architecture or VEGF axis dysregulation. Several immunological pathways previously associated with gene therapy response were upregulated, including engagement of adaptive immune response, already known to develop from ocular and systemic rAAV vector dosing. The inflammatory signatures were dose-dependent with most pronounced upregulation in the anterior region at high doses, followed by the retina, and mostly absent in the choroid. Histological analysis demonstrated dose-dependent increase in mononuclear infiltrates, including CD4+ T-cells and CD20+ B-cells. To determine if these immune landscape signatures could be detected in non-ocular tissues, serum was also evaluated. In summary, IVT administration of Ixo-vec was well tolerated up to 4E11 vg/eye (HED 8E11 vg/eye) supporting the current dose selections for human wAMD patients in the LUNA trial. These data support the lower doses being explored as well as combination of immunosuppressive prophylactics to suppress formation of adaptive immunity and prevent the development of chronic inflammation for ocular gene therapy.

OR27

Understanding the immune response to adeno-associated virus vectors in the central nervous system

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Adeno-associated virus (AAV) is a prominent gene delivery system that has resulted in five US Food and Drug Administration (FDA) and two European Medicines Agency (EMA)-approved therapies with many more in pre-clinical and clinical trials. Although AAV is considered to have low immunogenicity compared to other vectors, host immune responses to AAV gene therapies are a leading hindrance to their widespread application. Immune responses observed in human AAV clinical trials were not predicted from preclinical studies in animals, specifically induction of capsid-specific T cells which cleared transduced cells leading to loss of transgene expression. Even less is known about immune responses to AAV when targeted to the central nervous system (CNS) particularly because studying local immune responses is difficult in clinical trials. Our group has observed induction of systemic capsid-specific immune responses and increasing CXCL10 in the cerebral spinal fluid (CSF) in a CNS directed clinical trial. As we are limited by the kinds of samples we can collect in clinical trials, we sought to explore the immune response to AAV in animal models where we can collect CNS tissue for direct analysis. We bilaterally injected the thalamus of C57BL6 mice with either AAV8 or AAVrh32.33, which has previously been shown to induce capsid specific T cells in mice. We used these capsids to deliver the alpha-1-antitrypsin reporter gene and bled the animals biweekly until they were sacrificed at 2- (for early responses) or 12-weeks (established responses) post-injection. Splenocytes were isolated at endpoint and IFNγ ELISPOT assays were run to measure peripheral capsid specific T-cell responses. At 2 weeks, animals in both groups were negative for an IFNγ ELISPOT response, which is likely too early to observe a specific T-cell response, whereas at 12 weeks, 2 out of 4 animals from the AAVrh32.33 group were positive, indicating an antigen-specific T-cell response to the immune stimulating capsid. Further, ELISPOT positive animals demonstrated lower transgene expression, measured by ELISA, over time compared to ELISPOT negative animals within the rh32.33 group. Local immune infiltration was measured by flow cytometry of immune cells isolated from perfused brains at 2- and 12-weeks post injection. Cellular infiltration in both vector treated groups at 12 weeks versus 2 weeks potentially suggests that AAV capsid induces immune cell recruitment to the brain parenchyma after CNS delivery. Vector genomes, histology, and cytokine analysis are forthcoming, as well as further analysis of additional timepoints, transgenes and injection routes in the CNS. This data could help us further understand both local and systemic immune response to AAV gene therapy in the CNS, thus providing a model for understanding responses observed in clinical trials as well as developing immune-modulating strategies to allow for safer and more effective therapies.

OR28

A redosable non-viral DNA vector platform for highly potent, durable, non-inflammatory gene transfer

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Efficient and safe delivery of genetic material into cells is critical for translating gene therapy into clinical practice. The increased knowledge acquired during the past years in the molecular biology and nanotechnology fields has contributed to the development of non-viral vector systems as promising alternatives to virus-based gene delivery. While non-viral vectors have advantages such as low toxicity and immunogenicity, there are several critical issues currently limiting the translation of non-viral vectors into clinical success. Here we describe a unique DNA molecule (HALO™ DNA) which directs intracellular interactions favoring nuclear uptake, nuclear retention, and immunomodulation which greatly increases the efficiency and durability of gene delivery, while decreasing potential immunogenicity. We demonstrate that distinct structural elements within HALO DNA facilitate nuclear uptake and help mediate the extremely high potency seen upon HALO DNA delivery *in vivo*. Following intravenous delivery of HALO DNA (0.6 mg/kg) encoding tissue non-specific alkaline phosphatase (TNALP), a therapeutic transgene for the treatment of hypophosphatasia, we demonstrate a 10,000-fold increase over endogenous levels of plasma ALP activity that is stable for more than a year after a second dose. This level of TNALP expression and stability exceeds that reported for an AAV8-based vector at $5x10^{11}$ vg/mouse. Potency is, at least in part, mediated by increased nuclear uptake as evidenced by quantitative confocal imaging studies on primary human hepatocytes using lipid nanoparticle (LNP) formulated HALO DNA. Surprisingly, the structural elements present within HALO DNA are also able to modulate immune responses to the transgene product. Expression of a cynomolgus monkey TNALP (cmTNALP) in the mouse using a DNA construct lacking these structural elements was highly inflammatory and lead to an adaptive immune response to the transgene product which temporally limited expression and prevented re-administration. However, use of DNA containing the structural elements found within HALO completely abrogated the development of immunity and allowed repeat administration and stable gene expression. Importantly, this effect was irrespective of the presence of pathogen-associated molecular patterns (PAMPs) such as CpG elements, or selection markers, within the DNA. While CpG depletion or protein engineering-based approaches that diminished the likelihood of inflammatory signally arising from ER stress also increased potency of the HALO platform, the primary determinant for immunogenicity was the presence of the unique structural elements present within HALO DNA. HALO DNA appears to modulate immunogenicity by interfering with activation of the cGAS-STING pathway, presumably through interactions with key chromatin-associated enzymes within the cell. Due to their small size, biocompatibility, biodegradability, and their ability to effectively deliver nucleic acids into cells, we have focused on coupling HALO DNA with cutting-edge LNP technologies. We show that LNP formulated HALO-DNA can be effectively delivered to mice and mediates high levels of very durable gene expression. HALO-LNP formulations are non-toxic, as assessed by evaluation of ALT/AST levels, and non-inflammatory, as assessed by evaluation of a panel of pro-inflammatory cytokines. HALO DNA is also easily produced in a scalable, low-cost manner, providing additional promise for the continued development of this differentiated non-viral DNA vector system.

OR29

Preclinical immunogenicity of synthetic mRNA vaccines for infectious diseases: mycobacterium tuberculosis and hepatitis B virus

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Prevention and treatment of communicable diseases remains a global public health priority, particularly for infectious agents like *Mycobacterium Tuberculosis* (M.tb) and Hepatitis B virus (HBV) which are associated with high rates of morbidity and mortality. In Sub-Saharan Africa both M.tb and HBV remain endemic despite active preventative vaccination programmes, a situation that is further exacerbated by HIV-coinfection. Improved antigen-specific cellular immunity using mRNA-based platforms may enhance immunization and therapeutic strategies for these diseases. Consequently, we are studying the preclinical immunogenicity of lipid nanoparticle formulated synthetic mRNA and self-amplifying RNA (saRNA) vaccines for infectious diseases.
M.tb mRNA vaccine candidates were designed using rational antigen selection based on T cell receptor profiling (GLIPH2 similarity groups) from individuals who were able to control M.tb infection versus those that progressed to tuberculosis. In vitro transcribed modified mRNAs encoding sequence optimized CFP-10, WbbL1, PE-13 and PPE-18 proteins were co-transcriptionally capped and formulated using microfluidics. Markers of immunogenicity were measured in BALB/c and C57BL/6 vaccinated mice. Secreted cytokine profiles (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+). These results provide evidence of M.tb antigen-specific immunogenicity of mRNA vaccines in two different strains of mice.

In the case of HBV, synthetic saRNA vaccine candidates based on the Venezuelan equine encephalitis virus (VEE) replicon were generated. To improve immunogenicity and reduce the likelihood of vaccine non-responders, epitopes from HBV large (L-HBsAg) and small surface antigen (S-HBsAg) were included. To improve saRNA yields and reduce degradation, in vitro transcription was first optimized for longer transcripts. saRNA encoding HBV antigens or reporter proteins were initially evaluated in vitro, to determine if inherent interferon-stimulation compromised antigen translation. INF-β, IFIT1, PKR and OAS1 expression was measured by qRT-PCR, which showed stimulation of the innate immune response in a dose-dependent manner. However, ELISA-based analysis confirmed expression of HBV antigens from vaccine candidates even at the highest dose. Microfluidics was used to formulate saRNA within ionizable lipid nanoparticles and BALB/c mice were vaccinated using a prime-boost schedule. Bioluminescent imaging was used to track the in vivo expression kinetics of LNP-formulated saRNA encoding firefly luciferase. Spleens were isolated from vaccinated mice and ex vivo T cell stimulations showed an antigen-specific increase in CD8+ T cells expressing IFN- γ. Studies to determine correlates of protection are currently underway.

In conclusion, mRNA platforms may provide complementary or improved prophylactic and therapeutic vaccination strategies for both bacterial and viral infections, particularly in low and middle income countries which disproportionately bear the highest burden of communicable diseases.

OR30

Combinatorial knock-down/knock-out strategies to reconstitute antiviral immunity and eliminate persisting hepatitis B virus cccDNA

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Chronic hepatitis B virus (HBV) infection is a worldwide health problem which can lead to liver cirrhosis and hepatocellular carcinoma. HBV can establish a persistent form of its genome in the nucleus of the infected cells, the so-called covalently closed circular DNA (cccDNA). cccDNA acts as a transcriptional viral template and is a main source of constant viral antigen production. There are available therapy approaches for persistent HBV infection, such as pegylated interferon alpha and nucleoside analogues; however, they rarely eradicate the virus. Therefore, novel therapeutics are urgently needed to cure persistent HBV infection. Since a strong and polyclonal T-cell response is a robust predictor for viral clearance during acute infection, reconstitution of T-cell functionality by using novel approaches in chronic HBV infection is a promising therapy. In persistent HBV infection, HBV-specific T cells are scarce and dysfunctional, exhibiting high expression levels of exhaustion molecules such as PD-1, TIM-3 and LAG3. The interaction of immune checkpoint molecules and their corresponding ligands leads to the attenuation of T-cell activation. Thus, the aim of this study is the knock-down of ligands for PD-1 and TIM-3 (PD-L1, PD-L2 and Galectin-9 (Gal-9)) by using short hairpin RNAs (shRNAs). Continuous expression of shRNAs with minimal toxicity can be achieved by utilizing Adeno-associated viruses (AAV) as a vector. Furthermore, different tropisms of AAVs allow for transduction of specific cells, such as non-parenchymal liver cells, like Kupffer cells, liver sinusoidal endothelial cells and stellate cells, or organs. The scope of this project involved designing and screening shRNAs for murine PD-L1, PD-L2, and Gal-9 for efficiency using a dual luciferase assay. Selected shRNAs for Gal-9, PD-L1 and PD-L2 showed a knockdown efficiency of up to 85%. Meanwhile, GFP-encoding vectors based on different AAV capsids, AAVrh10a2, AAV7p2, AAV8p2, AAV9a2, and AAV9a6, were produced, and their transduction rates were tested in different mouse cell lines derived from hepatocytes and macrophages (AML-12 and RAW 264.7) using fluorescence microscopy. AAVrh10a2 showed the best transduction rate for both cell types, which is why in vitro experiments were performed with this capsid. Following the successful shRNA and capsid screening, AAV/shRNA vectors were produced and used to transduce AML-12 and RAW 264.7 cells. As a result, AAVrh10a2/shRNAs robustly suppressed Gal-9 and PD-L1 expression in vitro. As a combinatorial approach, three shRNA targeting Gal-9, PD-L1 and PD-L2 were cloned under the U6, H1 and 7SK promoters, respectively, in the same AAV vector (AAV-TRISPR). The knock-down efficiency of the final construct was verified by dual luciferase assay. Due to the different promoters used, we were able to detect knock-downs of Gal-9 by 90%, PD-L1 by 80%, and PD-L2 25% induced by the AAV-TRISPR construct. In summary, triple knock-down of immune checkpoint receptor ligands by a single AAV vector could be a promising approach to combat persistent HBV infection.

OR31

First-in-human trial of systemic CRISPR-Cas9 multiplex gene therapy for functional cure of HIV

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A major barrier to curing HIV is viral latency, which is defined as the persistence of integrated proviral DNA that is replication competent. Although antiretroviral therapy (ART) effectively suppresses viral replication and prevents disease progression to AIDS, long-term treatment with ART does not eliminate latent HIV and people living with HIV (PLWH) on suppressive ART have significant ART-related side effects and non-AIDS-related comorbidities and malignancies. EBT-101 is a CRISPR Cas9/gRNA multiplex investigational product targeting latent HIV-1 for genome editing, which is delivered by an adeno-associated virus serotype 9 (AAV9-SaCas9.U6sgLTR-1.U6sgGagD). EBT-101-001 is a first-in-human clinical study to evaluate the safety and efficacy of in vivo genome editing for a latent viral infection that is being developed for functional cure of HIV-1. EBT-101 has been well tolerated in the first study participants.

EBT-101-001 is a first-in-human Phase 1/2 open-label, single ascending dose study to assess the safety, tolerability, biodistribution, and pharmacodynamic effects of EBT-101 in aviremic, immune competent participants on ART. Three dose levels (9.0E+11, 3.0E+12, and 1.5E+13 vg/kg) of EBT-101 will be evaluated in three participants per dose level. Enrollment in the open-label study is sequential and dose escalation is staggered for safety review. Safety is the primary objective of the study and participants will be followed for 48 weeks post-dose. Eligibility criteria include stable HIV suppression on ART with CD4+ T cells > 500 cells/uL for at least one year and low anti-AAV9 neutralizing antibody titers. Participants receive 7 days of immunosuppression with dexamethasone starting 24 hours before IV EBT-101 administration. At week 12 post-dose, qualifying participants may stop their ART in an analytical treatment interruption (ATI) with weekly assessments for adverse events, HIV viral rebound, and changes in CD4+ T cell count. After 48 weeks, study participants will continue to be followed in a long-term follow up study for 15 years.

There have been no serious adverse events (SAEs) or dose limiting toxicities following EBT-101 administration in the first participants in the low dose cohort. There were 12 mild and reversible adverse events (AE) in the first two participants who have been followed for at least 26 weeks. Laboratory safety findings included transient elevations in ALT and AST. There were no changes in platelets, complement factors C3 and C4 or changes in troponin levels that have been associated with AAV9 delivery. Assessments of immunogenicity responses, biodistribution of EBT-101, detection of provirus excision and changes in the latent reservoir of HIV are being collected for analysis at the completion of the first cohort of participants.

The preliminary safety in the first two participants is encouraging and supports further clinical development for a fundamentally different approach toward functional cure of HIV.

OR32

Rapid generation of clinical grade personalized viral vectored vaccines encoding neoantigens for cancer immunotherapy

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Personalized vaccines targeting tumor neoantigens hold promise among next-generation cancer therapies. Reliable and fast process for vaccine production and quality control is mandatory for the success of this approach. Nous-PEV is a viral vector-based cancer vaccine encoding 60 patients' specific neoantigens identified by NGS of tumor biopsies and selected by Nouscom algorithm "VENUS". Synthetic cassettes encoding for identified neoantigens are cloned into Great Ape Adenovirus (GAd) and Modified Vaccinia Ankara (MVA) vectors, used in a Phase-I trial for prime/boost vaccination of patients with advanced Melanoma in combination with Pembrolizumab (NCT04990479). We optimized a robust manufacturing and quality testing process for parallel production of GAd and MVA vaccines, to release the final products at clinical site in 8 and 12 weeks respectively from the collection of the biopsy. Pilot GAd- and MVA-PEV lots were produced and fully characterized to assess throughput, quality, efficiency and reproducibility of the vector cloning and production procedures, before starting the clinical trial. Vaccines' production was completed in the expected timeframe for 11 out of 12 vaccines, designed to treat 6 patients enrolled in the study, supporting the feasibility and reliability of the whole manufacturing process. Subjects were treated according to the established schedules, with one patient only receiving MVA vectors; the combination treatment was safe and well tolerated. Ex-vivo interferon-gamma ELISpot on PBMC confirmed vaccine immunogenicity in 100% of evaluable patients (n=4), and this correlated with clinical response. This data supports feasibility and fosters future development of personalized cancer therapies based on recombinant viral vectors.

OR33

Selection of enhanced oncolytic and gene therapy vectors by Adenovirus Directed EVOlution (ADEVO)

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Directed evolution of novel viral vectors involves the generation of a random library of variants followed by the selection of novel improved variants according to pre-defined criteria. Directed evolution yielded few successes in Adenovirus vector (AdV) development until now, mainly due to insufficient diversity of random libraries.

AdVs clinical applications as gene therapy or oncolytic vectors are still hampered by the predetermined tropism of natural serotypes. To overcome this challenge, we hypothesized that validated vector retargeting technologies such as short peptide insertions on the vector surface

can be incorporated into the AdV toolbox. Here we designed novel Adenovirus Directed EVOlution (ADEVO) protocols for AdV retargeting, based on fiber knob peptide display.

As a proof-of-concept, AdV genome libraries were constructed by recombination of random oligonucleotides in the fiber gene, following three distinct protocols of library construction. Genome libraries were transfected in HEK293 cells to produce vector libraries, which were then selected by serial passaging on A549-ΔCAR cells that are non-permissive for natural Ad5, with the goal of identifying variants able to infect and lyse these cells efficiently. Library complexities were estimated by NGS variant calling and capture-recapture population size modeling.

All three ADEVO protocols enabled the construction of high complexity libraries, with up to 8.7e5, 9.6e5 and 1.7e5 unique variants in the respective initial vector libraries, which is an approximate 100-fold improvement compared to previously published AdV libraries. After 8 rounds of selection by serial passaging of the replication-competent vectors, the non-functional variants containing stop codons had been eliminated, and the most highly enriched variants were recloned and purified. Interestingly, they did not display enhanced or more specific transduction but more efficient cell lysis. This is consistent with the selection criteria given that variants were selected for the efficient completion of full replication cycles. Furthermore, it warrants investigations into potential unsuspected involvement of the fiber protein in late stages of the AdV replication cycle.

In order to enlarge the ADEVO toolkit, additional libraries were constructed based on non-Ad5 AdV types as well as Ad5 backbones detargeted for CAR, integrin and Factor X. In addition, we are currently optimizing a selection protocol specifically for gene delivery, whereby vector genomes internalized in target cells are purified shortly after transduction and their fiber inserts are recloned into a new library, ensuring that selection focuses solely on the cell entry step.

ADEVO, our novel directed evolution workflow for Adenoviruses, facilitates the construction of highly diverse libraries and the selection of improved vectors with user-friendly protocols. This new technology may contribute to the development of clinically relevant AdVs for a wide range of applications if utilized with stringent selection protocols optimized for the desired target cells or tissues.

OR34

Improving safety and productivity of rAdV manufacturing process by versatile adaptation to scale-X manufacturing technology

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The need for safer, more productive and robust processes are well-known challenges in development an infection-based viral vector manufacturing process linked to virus amplification in adherent cell lines. These challenges historically derive from the use of Fetal Bovine serum (FBS) in media composition, which poses safety risks, and heavy manual operations in tissue-culture treated flatware, which has limiting scalability and decreased robustness. The aim of this study was to approach those challenges from multiple angles and optimize the reference manufacturing process of VALO-D102 oncolytic recombinant Adenovirus (rAdV) in adherent A549 cells. We have utilized a Design of Experiment (DoE) statistical approach, to build a model of main process parameters, potentially linked with infectious rAdV particles. We have identified Serum-free media (SFM)

compositions, compatible with cultivation of A549 cells in suspension without presence of FBS. Also, we have adapted the adherent manufacturing process of A549 cells, previously performed in the flatware to the scale-X bioreactor system, which is a scalable manufacturing platform used for both adherent and suspension-adapted cells. During the DoE experiment, from the 30 conditions, tested in the DoE model (RSq=0,96 and PValue=< 0,0001), we have identified three key process parameters and their combinations, which in the best conditions, had achieved 3,8 times higher productivity (infectious units per cm^2), comparing to the reference process. Comparable productivity increase (2,3 times) from reference process, was maintained after application of the best DoE conditions to scale-X technology at 2,4 m2 Hydro bioreactor scale. Additionally, we have identified 2 compositions of serum-free media which supported successful adaptation of adherent A549 cells to cultivation in suspension mode. Those results open an opportunity to further advance development of large-scale manufacturing process rAdV manufacturing process with increased productivity, reached by DoE experiments and maintained in scale-X adaptation and increased safety, reached by successful adaptation of A549 cells to serum-free media composition. Combination of suspension adapted A549 cells, cultivated in SFM conditions, and identified key process parameters applied to scale-X fixed-bed bioreactor technology is an innovative approach for rAdV manufacturing . This approach will help to bring down the cost of goods of the future therapy, while increasing safety and robustness.

OR35

Self-amplifying RNA vectors encoding interleukin-12 armed with PD-1/PD-L1 blocking nanobodies induce potent antitumor responses

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Immunostimulatory cytokines are promising therapeutics for cancer therapy; however, their use is often hampered by a limited efficacy and an unfavorable toxicity, especially when they are administered systemically. Different strategies can be used to optimize their therapeutic potential, such as local administration to concentrate their action in the tumor site, or protein engineering to increase tumor targeting or provide additional effector functions. Here, we developed cytokines "armed" with antibodies, i.e. immunocytokines (ICKs), by fusing mouse interleukin-12 (IL-12) with nanobodies that block the interaction between mouse and human PD-1 and PD-L1. These ICKs were designed from either naturally occurring double-chain IL-12 (dcIL12) or from a synthetic single-chain IL-12 version (scIL12), and were fused to one or two nanobody copies by flexible linkers. The different constructs were cloned into a self-amplifying RNA vector based on Semliki Forest Virus (SFV) to evaluate their antitumor potential. Vectors based on SFV present characteristics that are attractive for cancer gene therapy, since they are able to induce a high and transient level of expression of the therapeutic transgene while inducing potent adjuvant effects due to the RNA self-amplification, which leads to strong inflammation and apoptosis in infected cells. First, correct expression of the different ICKs was confirmed in vitro by infecting cells with SFV vectors encoding these chimeric proteins. Similar levels of secreted ICKs and control IL-12 were achieved in all cases. The preservation of the biological functions of both components of the ICKs was confirmed by PD-1/PD-L1 binding assays, and by activation of mouse primary splenocytes in vitro. To evaluate their antitumor effect, ICKs were delivered locally into tumors using SFV viral vectors in a mouse model of colon adenocarcinoma. In this model, SFV vectors

encoding ICKs fused to anti-PD-1 nanobody (Nb11) showed a more potent antitumor effect than vectors encoding the cytokine alone or the ICKs carrying anti-PD-L1 nanobody. In terms of safety, local administration of these vectors did not induce toxicity in mice at the doses tested. One ICK based on scIL12 (scIL12-Nb11) was selected for further evaluation. SFV vectors encoding scIL12 or scIL12-Nb11 led to an increase in the infiltration of $CD8^+$ T cells in tumors. Mechanistically, the stronger antitumor activity of scIL12-Nb11 compared to scIL12 could be partially attributed to the targeting of the ICK to PD-1^+ cells. Within the tumor context, these cells predominantly consist of antigen-experienced $CD8^+$ T cells, including a subset that is tumor-specific. The antitumor effect of scIL12-Nb11 was also confirmed by a non-viral delivery method of SFV self-amplifying RNA, since this approach could be more easily translated to the clinic. As a proof-of-concept, a plasmid carrying the SFV replicon encoding the ICK was delivered locally by intratumoral electroporation, demonstrating safety and efficacy. We believe that these new "armed" cytokines and the self-amplifying RNA vectors used for their delivery could have a strong potential for cancer immunotherapy. Finally, the ICKs described in this work could be readily humanized by substituting the murine cytokine by the human counterpart, making them clinically relevant agents.

OR36

Upscaled production of next generation CAR macrophages derived from human induced pluripotent stem cells for efficient anti-cancer immunotherapy.

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Macrophages are among the crucial innate immune cells regulating the tumor microenvironment. Given macrophage plasticity, high capacity for tumor infiltration and known inflammatory functions, they have become attractive targets for chimeric antigen receptors (CARs). However, the efficient manufacturing of CAR macrophages from primary sources is challenging due to the low isolation yields of primary monocytes, and their resistance to the traditional gene editing methods. Thus, we showcase the scalable and continuous generation of CAR macrophages (CAR-iMac) from human induced pluripotent stem cells (hiPSC) as an alternative cell source. For proof of concept, hiPSC cells were genetically-engineered with an anti-CD19-CAR receptor. By employing our modern upscaled macrophage differentiation platforms, we could successfully show continuous CAR-iMac production of consistent quality and over extended time period, using either small (3mL) or intermediate scale (40mL) devices. Indeed, utilizing an intermediate scale automated bioreactor platform allowed for the weekly harvest of CAR iMacs with an average yield of ~

1×10^7 cells/week/40mL. The generated CAR-iMac showed typical macrophage morphology ($CD45^+$, $CD14^+$, $CD11b^+$, $CD163^+$), and stable CAR expression throughout the different stages of hematopoietic development. On a functional level, CAR-iMac had enhanced phagocytosis of $CD19^+$ B cell lymphoma cancer cells (Raji) with 75.9% of phagocytosis compared to 34.7% by control $eGFP^+$ iMac. Notably, the CAR receptor provided specificity to macrophages and mediated an antigen specific phagocytosis. While CAR-iMac and control cells were similarly phagocytosing $CD19^-$ K562 leukemia cells with an average of 6.8% and 11.3% of phagocytosis, respectively. However, only CAR-iMacs were efficiently eradicating two different leukemia cell lines (Raji, Daudi) in a CD19 expression dependent fashion. In addition to the enhanced phagocytosis, CAR-iMacs were superior to the control iMacs in secreting pro-inflammatory cytokines including IL-6 and TNFα upon the co-culture with the cancer target cells, providing another pivotal mechanism to enhance the anti-tumoral activity of the macrophages. To provide a clinical translation of our findings, we have evaluated the targeting capacity of CAR-iMac against primary acute lymphocytic leukemia (ALL) samples, which were recruited from five different patients. The ALL samples were of high purity to their B cell composition. Similarly to the observed effects with Raji cells, CAR-iMacs showed enhanced phagocytosis of $CD19^+$ ALL samples compared to control cells. Further scRNA sequencing revealed that CAR-iMac cluster distinctly from $eGFP^+$ iMac with upregulation of genes associated with M1 polarization (NF-kB1, IL-6) , adaptive immune cell recruitment (CXCL10,CCL5), and antigen presentation (HLA-A, HLA-DRB1), signifying the impact of the CAR in driving a pro-inflammatory milieu. By comparing the enriched GO terms on the top 100 differently-expressed genes we can further demonstrate that CAR-iMacs had a stronger activation of antiviral response as an additional novel insight into the CAR iMac defense mechanism against the cancer cells. Of note, clearance of Raji or ALL samples followed similar signaling pathways in CAR-iMac. In conclusion, we present a modern, scalable and robust platform for continuous generation of functional CAR-iMac, which is of crucial importance to provide access to the next generation of off-the-shelf immune cell-based therapies.

OR39

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 *GBA1* transgene that encodes an engineered variant of glucocerebrosidase (GCase-85) under control of a liver-specific promoter. GCase-85 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). The increased stability of GCase-85 may also increase its tissue coverage compared to ERT.

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 GCase-85 expression and substrate reduction. The starting dose of FLT201 for the first cohort is $4.5x10^{11}$ vector genomes per kilogram bodyweight, with subsequent dose escalation based on observed safety and efficacy. Participants will be followed for 38 weeks after treatment before entering long-term follow-up. The first participant, a 35-year old male on enzyme replacement therapy, has been dosed and tolerated the infusion well. No adverse events have been reported for this participant at this time. Tests at screening showed GCase activity (plasma) of 0.07 micromol/L/h, GCase activity (dried blood spot) of 0.3 micromol/L/h, and Lyso-Gb1 (dried blood spot) of 102.85 ng/mL. Study data from participants enrolled into the first dose cohort, including patient demographics, safety and GCase levels, will be presented.

OR40

Sustained LCAT replacement in patient with familial LCAT deficiency in clinical trial of *ex vivo* gene/cell therapy using autologous preadipocytes

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Familial lecithin:cholesterol acyltransferase (LCAT) deficiency (FLD) syndrome is an autosomal recessive disease characterized by low plasma LCAT activity and severe dysfunction of HDL with subsequent impaired lipoprotein metabolisms. Patients often develop complications such as corneal opacity, hemolytic anemia, and renal disease. In plasma protein deficiency such as LCAT deficiency, gene therapy-mediated continuous enzyme replacement is the most suitable therapeutic approach. We have postulated adipocyte-based *ex vivo* gene therapy-mediated enzyme replacement therapy (ERT), in which genetically-modified adipocytes (GMAC) reside at transplanted sites to continuously supply therapeutic proteins. We have applied our GMAC technology to treat FLD via transplantation of *LCAT* gene-transduced autologous adipocytes (LCAT-GMAC). The *ex vivo* gene therapy protocol has been approved under the Act on Securement of Safety of Regenerative Medicine in Japan. First patient was enrolled in January of 2017, and safety as well as efficacy has been investigated, and the clinical evaluation of the prescribed observation period and follow-up period (total of 240 weeks after administration) was completed in the patient. No replication-competent retrovirus in subject's blood sample or tumorigenicity of the LCAT-GMAC was observed. Post-administration pain was recognized, however, no abnormalities were observed in other clinical parameters in the patient. Increased serum LCAT activity has sustained for 240 weeks. Together with changes in laboratory values related to lipid metabolism, the long-term amelioration of abnormal lipoprotein metabolism was suggested in the patient. The hemoglobin-haptoglobin complex produced with hemolytic anemia was also reduced, which suggested improved vulnerability of erythrocytes. Thus, LCAT-GMAC is possible curative ex vivo gene/cell therapy product for FLD patients. The GMAC technology will provide a novel platform for *ex vivo* gene therapy mediated ERT for variety of intractable diseases.

OR41

Molecular, cellular and clinical implications of lentiviral-mediated gene therapy in patients with Fanconi anemia

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The phase I/II clinical trial in patients with Fanconi anemia A (FA-A) (FANCOLEN I) has shown the progressive engraftment of corrected hematopoietic stem and progenitor cells (HSPCs) in most patients, in the absence of any pre-conditioning regimen. Here we report the results corresponding to the FANCOLEN I and long-term follow-up (LTFU) trial up to 7 years after infusion of transduced HSPCs. Additionally, we show data revealing the potential of gene therapy to revert the transcriptional program of FA-HSPCs. Non-conditioned FA-A patients were infused with mobilized $CD34^+$ cells that had been transduced with the PGK-FANCA.Wpre* lentiviral vector. At sequential times post-infusion, peripheral blood (PB) and bone marrow (BM) samples were obtained to assess the efficacy and safety of gene therapy at a molecular, cellular and clinical level. Six of the eight evaluable patients showed a progressive increase in the proportion of gene-corrected cells both in BM and PB, which correlated with the level of MMC resistance in BM HSPCs and also with the reduction in the chromosomal instability of PB T cells exposed to diepoxybutane. To evaluate the efficacy of gene therapy to revert characteristic biochemical pathways affected in FA HSPCs, single -cell RNA sequencing (scRNAseq) studies were conducted in corrected and uncorrected BM HSPCs in four gene therapy treated FA patients. Single-cell RNA sequencing in HSPCs showed for the first time in a gene therapy trial that gene complementation can revert the transcriptional signature characteristic of diseased HSPCs, which then resembled the transcriptional program of healthy donor HSPCs. This included a downregulated expression of TGF-β and p21, as well as an upregulation of the DNA damage response and telomerase maintenance pathways. Long term follow-up studies showed that infusion of low numbers of corrected HSPCs in patients with relatively advanced BMF could not overcome the progression of the BMF since the progression of the disease was faster than the engraftment of corrected HSPCs. In these cases, alternative treatments including transfusions and/or allogenic transplantation were provided. In contrast to this observation, patients treated in early stages of BMF with higher numbers of corrected HSPCs showed sustained engraftment of corrected HSPCs, and even progressive recovery of BMF. The results obtained in the FANCOLEN I trial reveal that gene therapy can revert the transcriptional program in HSPCs from FA patients, leading to the cellular and molecular phenotypic correction of the entire hematopoietic system of these patients. Moreover, the LTFU trial shows that gene therapy conduced in early stages of the disease can revert the progression of the BMF. Based on these observations, a global phase II trial is currently ongoing in FA patients in early stages of the disease under the sponsorship of Rocket Pharmaceuticals Inc.

OR42

Efficient and specific *in vivo* genetic engineering of human hematopoietic stem progenitor cells without selective conditioning

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Achieving *in vivo* genetic engineering of hematopoietic stem/progenitor cells (HSPCs) would transform treatment for hematological disorders. Stably humanized mouse models carry a highly skewed human hematopoiesis and are not well suited for establishing the efficacy of HSPC-targeted gene therapy platforms. We developed a multi-model strategy to assess efficiency and specificity of access to human HSPCs and achieved efficient genetic engineering of Lin-CD34+ cells and individual HSPCs subtypes without selective enrichment using highly potent lentiviral (LV) and editing viral like particles (VLP). To establish peripheral blood (PB) access to HSPCs, we followed long-term NBSGW mice where BaEVTR-LV was dosed intravenously (IV) right after HSPC infusion, showing targeting of 23% early-engrafting bone marrow (BM) HSPCs and stable 5-7% engineered PB myeloid output up to wk16. To investigate BM access, we dosed BaEVTR-LV at D7 post-humanization, when human HSPCs have physiological composition, are few and are confined in the BM, reaching 5% of BM-resident HSPCs with a single IV dose. Based on these data we developed a potent BaEVTR VLP carrying CRISPR/Cas9 editor targeting the B2M locus. This VLP displayed ~92.5% editing efficiency in unmanipulated HSPCs *in vitro* and, when dosed *in vivo* in the D7 post-humanization model, achieved a remarkable 31% B2M KO in BM-resident Lin-CD34+ cells. We are now combining this VLP with a CD133 retargeted fusogen. We will present unpublished high-resolution data showing that our CD133 fusogen-LV combines potency *on par* with BaEVTR-LV *in vitro* and *in vivo* with a remarkable specificity for CD133+ HSPCs avoiding multiple human cellular “sinks”.

OR43

Non-genotoxic conditioning and *in vivo* gene transfer as new therapeutical approaches for autosomal recessive osteopetrosis

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Autosomal recessive osteopetrosis (ARO) is a rare disease characterized by defects in osteoclast differentiation or resorption, having an incidence of 1:250.000 live births. Mutations in *TCIRG1* gene, encoding the V-ATPase proton pump involved in bone resorption, cause osteoclast-rich osteopetrosis, characterized by dysfunctional osteoclasts. Hematopoietic stem cell transplantation is the standard of care for these patients, although approaches to improve disease-free survival are needed. New non-genotoxic conditionings are being developed to limit the side effects of conventional conditioning regimens and to achieve better transplant outcomes. A promising antibody-drug conjugate is formed by the Saporin (SAP) toxin, a catalytic N-glycosidase ribosome-inactivating protein that interferes with protein synthesis, conjugated to an anti-CD45 antibody, to specifically target hematopoietic stem and progenitor cells (HSPCs). We tested CD45-SAP in the *oc/oc* mice, the murine model of *TCIRG1*-osteopetrosis, to deplete HSPCs sparing the other cell types. We conditioned newborn mice at day 1 or 2 of life with CD45-SAP and, after 48 hours, we transplanted them with $7*10^6$ CD45-mismatched WT bone marrow (BM) cells. In untransplanted animals, CD45-SAP depleted HSPCs in BM and at a lower extent in the liver, while limited efficacy was found in spleen and peripheral blood (PB). Upon conditioning and transplantation, bone defects were rescued in *oc/oc* mice. Notably, CD45-SAP-treated *oc/oc* mice engrafted a higher fraction of CD45.1 cells in PB and BM (mean of 6% on live cells), spleen (mean of 4% on live cells) and thymus (mean of 3% on live cells), than CD45-SAP-treated WT mice, without perturbation of the immune subset distribution. Two out of 6 *oc/oc* mice survived long-term (20w), beyond their expected life span (2-3w). At termination, we cultured BM and spleen cells on dentine slices and differentiated them into osteoclasts. We could detect resorption pits by toluidine blue staining. As an alternative strategy for the treatment of *TCIRG1*-ARO, we tested *in vivo* lentiviral vector (LV) gene therapy (GT). At birth, *oc/oc* mice were injected *in vivo* intravenously with a LV expressing the *TCIRG1* gene under the control of phosphoglycerate kinase (PGK) promoter. To evaluate transduction efficiency, we first measured vector copy number (VCN) in PB, BM, spleen, thymus, liver and brain. Despite a generally low transduction efficiency, we observed higher VCN in *oc/oc* mice (1.5% of marking in BM and SPL) than in WT or heterozygous LV-treated littermates. The highest VCN was detected in the liver (10% of marking), as expected. LV-treated *oc/oc* mice showed lower frequency of B cells in PB and spleen, with higher percentage of myeloid cells in spleen and T cells in BM, if compared to control mice. These differences are probably due to the limited space in the BM niches typical of the *oc/oc* model. Importantly, GT *oc/oc* mice survived longer than untreated, up to18w. Preliminary results show normal parameters of bone architecture in *oc/oc* GT mice comparable to healthy littermates. In conclusion, we observed that non-genotoxic CD45-SAP conditioning allows engraftment of donor cells in the osteopetrosis setting. Moreover, *in vivo* LV-gene transfer may represent an alternative treatment for *TCIRG1*-osteopetrosis.

OR44

Development of gene therapy for dermatosparaxis Ehlers-Danlos syndrome

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Dermatosparaxis Ehlers-Danlos syndrome (dEDS) is an autosomal recessive connective tissue disorder caused by a loss of function mutation in ADAMTS2 encoding for procollagen N-proteinase. The mutation impairs the cleavage of the N-propeptide from type I procollagen chains resulting in mature collagen fibrils with aberrant morphology and lower tensile strength. Patients with dEDS present with severe skin fragility and redundant skin, hernias, craniofacial deformities, short stature, easy bleeding and bruising, premature placental rupture of the membranes, and visceral fragility. This type of EDS was selected for development of 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) ADAMTS2 is ubiquitously expressed throughout the body. Due to the extracellular action of ADAMTS2, AAV9 was selected with the goal of transducing liver and skeletal muscle to provide stable long-term expression.

Four AAV9 vectors encoding for a wild type signal peptide or an enhanced signal peptide, as well as with an HA tag located at the C-terminus of the cDNA or in the middle of the cDNA were screened for toxicity in 6-week-old C57BL/6J mice. Mice in each group (n=4) were treated via intravenous tail vein injection at a dose of 1E12 vg/mouse. Mice were sacrificed one month later. The toxicity study produced no adverse clinical outcomes in any mice, and tissues appeared grossly normal at necropsy. Histopathology of skin, aorta, quadriceps femoris, lung, liver, kidney, brain, and spinal cord were normal. Transmission electron microscopy of the skin and aorta were also normal. Western blot demonstrated the increased expression of an active cleavage form of ADAMTS2 plus the HA tag at approximately 75 kDa for the vector utilizing the wild type signal peptide and with the HA tag on the C-terminus of the cDNA. Other bands were seen for all vectors at approximately 45 kDa and 20 kDa, likely representing other cleavage products of ADAMTS2. Vector genome biodistribution by qPCR is underway.

Following this, the top three vectors were screened in *Adamts2*^{KO} (n=6) and normal littermate controls (n=6) at 1E12 vg/mouse via intravenous tail vein injection. Mice were treated post-symptomatically at 8-weeks-of age and AAV treated animals are thus far 6 months of age. We will employ two endpoints, 8 months and 4 months, to evaluate short- and long-term expression.

AAV treated *Adamts2*^{KO} mice- have thus far not exhibited adverse clinical signs, including those associated with dEDS. Endpoint is scheduled for Fall 2023. Best vector selection will be based on normalization of collagen morphology by transmission electron microscopy as well as biochemical electrophoretic analysis of procollagens. We will also perform RT-qPCR analysis of ADAMTS2 mRNA expression, western blot for HA tag and vector genome biodistribution by qPCR. Following this we will initiate a clinical trial in pet dogs suffering from a naturally occurring form of dEDS. Efficacy from this study will be used to support first in human AAV gene therapy trial for dEDS.

OR45

Exploring actionable strategies to improve AAV5-hFVIII-SQ durability and optimize gene expression

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Roctavian (AAV5-HLP-hFVIII-SQ) is an approved gene therapy in the EU for the treatment of severe hemophilia A. A single infusion provides therapeutic levels of FVIII expression in adult men. The precise mechanisms contributing to variability and durability in transgene expression are unknown. Multiple lines of evidence suggest low RNA production contributes to the decline of FVIII expression and low-response to AAV gene therapy. Previously, we have shown that decreased interaction of active histones with episomal genomes may mediate the decline in transgene expression. Additional studies suggested hepatocyte capacity to fold and secrete FVIII may contribute to variability. We hypothesize (1) modifying the chromatin interaction with AAV-episomes using epigenic regulators may increase accessibility of vector genomes and (2) the use of molecular chaperones may improve FVIII folding and secretion. Using in vitro hepatocyte models, epigenetic modifiers and molecular chaperones were screened to evaluate effects on FVIII RNA and secreted protein levels. We identified a molecular chaperone capable of significantly increasing FVIII protein secretion. Furthermore, three epigenetic modifiers significantly potentiated (> 10-fold) and re-activated FVIII RNA expression (>40-fold) following drug-induced transgene silencing in vitro. Subsequently, we tested a histone deacetylase inhibitor in vivo. Significantly increased AAV-mediated transgene expression was observed in mice treated with the histone deacetylase inhibitor, at much lower doses than indicated for currently approved use. These results suggest the potential use of epigenetic modifiers to enhance AAV-mediated expression and reactivation, if silenced; and the use of chemical molecular chaperones to improve FVIII secretion; thereby optimizing the hemostatic benefit for hemophilia A patients.

OR46

Lentiviral gene therapy reverts GPIX expression and phenotype in Bernard-Soulier syndrome type C

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Bernard-Soulier syndrome (BSS) is a rare congenital disorder characterized by a reduced platelet count of unusually large size, leading to frequent bleeding episodes and severe haemorrages. It is caused by specific pathogenic variations in three genes: *GP1BA*, *GP1BB*, or *GP9*. These genes encode the GPIbα, GPIbβ, and GPIX subunits of the GPIb-V-IX complex, which serves as the primary receptor on platelet surfaces for von Willebrand factor. This complex plays a crucial role in recognizing vascular injury zones and facilitating platelet recruitment, activation, and aggregation. BSS is classified into three types, A1 (*GP1BA*), B (*GP1BB*), or C (*GP9*), this last being associated with the highest number of pathogenic variants reported to date. Mutations in these genes disrupt the assembly of the GPIb-V-IX receptor, resulting in a hemorrhagic phenotype. Currently, the available measures to manage the disease include prevention, palliative treatments, and, in exceptional cases, allogenic transplantation of hematopoietic stem cells (HSCs). Unfortunately, patients with BSS face lifelong challenges that impact their quality of life.
Leveraging the CRISPR-Cas9 technology, we have developed *GP9*-knockout (*GP9*-KO) disease models to investigate receptor biology in megakaryoblastic cell lines. Additionally, we have replicated the differentiation process that HSCs undergo to produce aberrant megakaryocytes and oversized platelets by knocking out *GP9* in induced pluripotent stem cells (iPSCs).

To address the impaired assembly of the GPIb-V-IX receptor, we have developed innovative self-inactivating lentiviral vectors. These vectors enable specific expression of GPIX in megakaryocytes, thereby correcting the expression and localization of GPIX and reassembling the entire GPIb-V-IX receptor. This restoration of functionality allows the receptor to bind von Willebrand factor effectively. Furthermore, by transducing *GP9*-KO iPSCs and subsequently inducing differentiation, we have successfully reversed the oversized platelet phenotype and restored the expression of the GPIb-V-IX receptor.

Moreover, we have conducted gene therapy experiments using hematopoietic stem cells obtained from two unrelated patients with BSS type C carrying missense mutations. By transducing these cells with our gene therapy vectors and guiding their differentiation into megakaryocytes, we have achieved GPIX expression. Consequently, the resulting platelets displayed a reduced size.

Through our study, we aim to demonstrate the efficacy of this promising gene therapy approach in treating conditions such as BSS. Our findings underscore the potential of gene therapy as a viable

treatment option for Bernard-Soulier Syndrome type C, opening new possibilities for addressing platelet disorders.

OR47

Genetic engineering of natural killer cells for off-the-shelf cell therapy strategies against cervical cancer: targeting Mesothelin and Fibroblast activation protein

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Genetic engineering of immune cells for use as living drugs has garnered significant attention and demonstrated remarkable success in treating liquid cancers, particularly B-cell leukaemia & lymphoma, by equipping T cells with chimeric antigen receptors (CAR). Expanding upon this approach, there is immense potential to revolutionise the treatment landscape for solid tumours by leveraging novel CAR-based immunotherapies that harness the patient's immune system. Cervical cancer, the most prevalent gynecologic malignancy, poses a significant health burden world-wide with high incidence (>600,000 cases annually) and mortality (>300,000 cases annually) within gynecologic oncology. Conventional treatment options, including radical hysterectomy, chemotherapy, and radiotherapy, often yield limited responses in late-stage cervical cancers, highlighting the urgent need for innovative therapeutic approaches. In light of this, our study aims to develop off-the-shelf cell therapy strategies against cervical cancers utilising genetic engineering techniques.

Flow cytometric-based antigen expression screens identified Mesothelin as a promising target antigen that exhibits high expression on cervical cancer cells. Additionally, fibroblast activation protein (FAP) was frequently detected in primary cervical cancer-associated fibroblasts, highlighting its potential as a target within the tumour microenvironment (TME). Given the intrinsic MHC-independent anti-tumour activity of natural killer (NK) cells and their suitability for off-the-shelf cell therapy approaches, we used self-inactivating (SIN) alpharetroviral vectors to genetically engineer NK cells with third-generation chimeric antigen receptors (CAR) with the aim to specifically eliminate antigen-positive cervical cancer cells using an approach that can be translated for clinical application.

We employed state-of-the-art fluorescence-based techniques, such as flow cytometry and live-cell imaging, to assess the cytotoxicity of anti-FAP and anti-Mesothelin CAR-NK cells. Our evaluation encompassed both monolayer (2D) and spheroid (3D) models, demonstrating the efficient killing capacity of anti-Mesothelin and anti-FAP CAR-NK cells against established cell lines (*e.g.*, SiHa, HeLa) and primary cells isolated from patient-derived tumour samples. To confirm the specificity of anti-Mesothelin CAR-NK cells, we utilised CRISPR-Cas9-mediated knockdown of Mesothelin in target cells. This approach served as an additional negative control, further validating the target specificity of anti-Mesothelin CAR-NK cells. Additionally, we explored the combinatorial cytotoxic effects of anti-Mesothelin CAR-NK cells by investigating their interaction with clinically used chemotherapeutics, including cisplatin.

The results of our study hold great promise as potential treatment options for cervical cancer patients, as well as the potential for extension to other gynaecological malignancies and fibrotic diseases. Our data support the feasibility of cell immunotherapies to improve the management and outcomes of patients afflicted with these challenging conditions.

OR48

Harnessing the adenosine pathway by genome engineering to enhance the functionality of TCR-edited T cell products

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In solid malignancies, tumor-infiltrating lymphocytes (TILs) functionality is closely intertwined with metabolic reprogramming within the tumor microenvironment (TME), which supports the increased energy request of cancer cells and suppresses the effector function and cytokines production of the immune infiltrate. In primary colorectal cancer (CRCs) and related liver metastases, we revealed an extensive transcriptional remodeling across tumors, being metabolic pathways among the major drivers of this variance. Accordingly, extracellular adenosine, accumulating in the TME for the sequential hydrolysis of ATP by ectonucleotidases CD39 and CD73, has been recently recognized as a novel inhibitory mediator, which binds preferentially to the A2A receptor (A2Ar) to exert its immunosuppressive activity. To unveil whether the inhibitory profile of T cells infiltrating CRCs and derived liver metastases is related to the adenosine pathway, we used high dimensional flow cytometry coupled with a complex pipeline of dimensionality reduction and clustering algorithms. By comparing TILs with T cells infiltrating peritumoral and non-neoplastic colon and liver tissues, we observed a strong co-expression of multiple inhibitory receptors (IRs), with CD39 emerging as the major driver of exhaustion in TILs from both primary and metastatic colorectal tumors. Therefore, we sought to counteract the immune suppressive TME by disrupting CD39 in TCR-edited T cells. We leveraged on the multiplexing capacity of the CRISPR/Cas9 system to contemporary disrupt both the α and β chains of the endogenous TCR together with *ENTPD1*, the gene encoding for CD39, and we redirected T cell specificity against the HER2 antigen by lentiviral transduction, employing a novel HER2 TCR that we isolated and reconstructed (TCR_{ED}). $TCR_{ED}CD39_{KO}$ T cells proved superior to their competent counterpart in eliminating patient-derived organoids (PDOs) from primary and metastatic tumors both *in vitro* and *in vivo*. Given the role of CD39 in starting the biochemical cascade that leads to adenosine production, we aimed at investigating the direct role of adenosine on the functional advantage observed with $TCR_{ED}CD39_{KO}$ T cells. We challenged our cellular products with target cell lines in the presence or absence of saturating concentrations of adenosine and observed that $TCR_{ED}CD39_{KO}$ T cells are superior to their competent counterpart in the ability to release cytokines, while the effect is completely abolished when adenosine is present. Also, when $TCR_{ED}CD39_{KO}$ T cells were challenged with PDOs, the exogenously added adenosine nullifies the functional advantage given by the CD39 disruption. To gain more insights into the pathway, we also generated and tested $TCR_{ED}A2Ar_{KO}$ T cells. In line with the different roles of CD39 and A2AR on the ATP/adenosine pathway, the disruption of the adenosine receptor resulted in a significant advantage in PDO killing

in the presence of adenosine. *In vivo* studies to compare $TCR_{ED}CD39_{KO}$ and $TCR_{ED}A2Ar_{KO}$ T cells in their ability to control tumor outgrowth are currently ongoing. Overall, we showed that the genetic disruption of adenosine-signaling genes in T cells to be adoptively transferred in CRC patients is a valuable strategy to target the tumor while rendering the cell product able to counteract the immune suppressive TME.

OR49

ROR2 is a novel cross-entity target for CAR-T cells in multiple myeloma and clear cell renal cell carcinoma.

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Chimeric antigen receptor (CAR) T cell therapy directed against CD19 or BCMA has achieved outstanding clinical results in hematological tumors. However, there is a desire to identify and validate novel cross-entity targets and to develop innovative CAR-T cell products, in order to broaden the therapeutic applicability of CAR-T cells. In this study, we were interested in evaluating the receptor tyrosine kinase ROR2 as a novel target for CAR-T cell therapy, due to its overexpression on the cell surface of various cancers.

To dissect the relevance of targeting ROR2, we performed RNAseq analysis of 27 cancer entities from the TCGA and COMPASS datasets. Our data show ROR2 to be overexpressed as compared to healthy tissue in at least 40% of cancer cases in 10 out of 27 entities, including three of the 10 most common cancers (breast, kidney and liver cancer). We also found ROR2 to be uniformly overexpressed in multiple myeloma (MM), and a strong correlation with disease-specific survival in clear cell renal cell carcinoma (ccRCC). Next, we employed qPCR, immunohistochemistry and flow cytometry to validate these *in silico* findings in representative patient cohorts and found ROR2 to be expressed in 80% of MM and 100% of ccRCC samples.

We generated 2nd generation ROR2-specific CAR-T cells (ROR2-CART) and analyzed their anti-tumor reactivity *in vitro* and *in vivo*. Our analyses revealed ROR2-CART to elicit potent antigen-specific tumor cell lysis, cytokine secretion and proliferation in response to ROR2-positive MM and RCC cell lines but not ROR2-negative tumor cell line. In line with these findings, ROR2-CART showed potent anti-tumor efficacy *in vivo* and led to a significant increase in median overall survival in xenograft models of MM and ccRCC (*p=0.0039* and *p=0.0035*, respectively). While ROR2-CART were able to cure 25% of mice in our MM model, all RCC xenografts relapsed and ultimately succumbed to the disease. Therefore, we decided to analyze whether transcription factor modulation could be employed to augment the anti-tumor efficacy of ROR2-CART. Our data show that the over-expression of cJUN in $CD4^+$ and $CD8^+$ T cells significantly improved the anti-tumor efficacy of ROR2 CAR-T against ccRCC both *in vitro* and *in vivo*.

Finally, we were interested in assessing the safety of targeting ROR2 in preclinical models. Our analyses show that mice are a relevant model to study the safety of targeting ROR2 with CAR-T cells, as well as cross-reactivity of our ROR2-CART with murine ROR2. However, no signs of toxicity or tissue damage were observed, when we inoculated tumor-free mice with up to $4x10^8$ ROR2-CART per kg.

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.

OR50

High affinity CAR-T cells exhibit increased resistance to PD-1/PD-L1-mediated inhibition

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Overcoming T-cell inhibition within the tumor microenvironment is a critical challenge for achieving optimal efficacy of CAR-T cell therapy in solid tumors. One potential strategy to address this issue is by disrupting PD-1 signaling in CAR-T cells. However, there have been conflicting findings regarding the effects of targeting this pathway using various CAR constructs. In light of these discrepancies, we hypothesized that the affinity of the CAR for its target antigen may be an important and unexplored factor modulating T-cell sensitivity to the PD-1/PD-L1 axis.
In this work, we interrogated the sensitivity of CAR-T cells targeting HER2 with low (LA) or high (HA) affinity to inhibition by PD-L1. By using a preclinical model of tumor cell lines engineered to express different levels of PD-L1, we observed that cytokine secretion by LA HER2-CAR-T cells gradually decreased as PD-L1 densities increased. By contrast, HA HER2-CAR-T cells displayed a lower degree of inhibition. Accordingly, CRISPR-mediated knockout of PD-1 restored effector functions of LA HER2-CAR-T cells *in vitro* and improved their performance *in vivo* in mice bearing tumors expressing variable or wild type levels of PD-L1. By contrast, PD-1 KO did not significantly impact functionality of HA HER2-CAR-T cells neither *in vitro* nor *in vivo*, where they were able to completely eliminate tumors even in the high PD-L1-expressing model. These differences between LA and HA CAR-T cells in regard to PD-1/PD-L1-mediated inhibition have been observed in CAR-T cells comprising CD28 or ICOS as co-stimulation domains, suggesting that are not unique for a particular CAR construct.

Finally, we explored the effects of PD-1 KO in CAR-T cells targeting other tumor antigens with varying affinities. Similar to the observations made in the HER2 CAR model, genetic disruption of PD-1 significantly improved antitumor activity of CAR-T cells targeting mesothelin with low affinity, while did not impact CAR-T cells targeting folate receptor alpha (FOLR1) with high affinity.

In conclusion, low affinity CAR-T cells are more sensitive to PD-L1-mediated inhibition as compared to those recognizing the antigen with high affinity. This inhibition can be overcome by genetic disruption of PD-1. Moving forward, further investigation is needed to fully elucidate the mechanisms underlying these observed differences.

OR51

Reconstruction of novel Adeno-associated virus (AAV) genome variants using metagenomic sequencing data

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The exponential expansion of metagenomic data obtained through High-Throughput Sequencing (HTS) technologies has surpassed the petabyte-scale threshold, resulting in an unprecedented abundance of data that now enables, in principle, the *in silico* discovery of previously unknown viral and bacterial species. To date, however, only a few studies have tapped into this enormous potential in the context of widely used gene therapy agents, such as the Adeno-associated viruses (AAV). Naturally occurring AAV variants exhibit distinct transduction efficiency in specific tissues or species, attributed to their evolutionary adaptation. Consequently, there is a compelling drive to employ HTS technologies and advanced computational capabilities in the quest to identify and characterize further naturally existing AAV variants. In this study, we demonstrate the power and promise of mining metagenomic databases to uncover unknown natural AAV rep(lication) and cap(sid) genes, with the dual aims of (1) expanding our repertoire of templates for vector development while (2) simultaneously enhancing our understanding of virus evolution across different species. Notably, a substantial amount of publicly accessible raw metagenomic data can be accessed through tools like the Serratus Explorer. The latter was harnessed here to identify 29 previously unknown AAV variant sequences in metagenomic data from birds, non-human primates or humans, of which 16 including three from human gut were classified as high-quality based on the high coverage of their *cap* region. To this end, we established a comprehensive computational pipeline that comprises (i) reference candidate selection, (ii) pre-aligned data acquisition, (iii) variant calling and frequency estimation, (iv) consensus calling, (v) variant resolution, (vi) phylogenetic analysis, and (vii) protein structure analysis. From this list, eight *cap* genes from four different species were synthesized and used to produce reporter gene-encoding AAV vectors. Results from their experimental characterization including measurement of gene transfer efficiency and reactivity with anti-AAV antibodies showcase the great promise and value of our approach for basic AAV research and gene therapy vector development. Furthermore, to assess the effectiveness of this reference-based variant reconstruction pipeline, we concurrently conducted a conventional, reference-independent metagenome-based genome assembly approach. The latter successfully reconstructed *rep* and *cap* genes of AAV variants, but solely for abundant variants representing the majority of aligned reads with shared mutations, thereby excluding other, less frequent variants within the same sequencing pool. Combined with the fact that this conventional method of genome assembly from large metagenomic sequencing files necessitates substantial computational resources and significantly extends the required processing time, this highlights the assets and superiority of our original consensus-based reconstruction pipeline.

The first three authors contributed equally

OR52

Retrograde renal pelvis injection of select AAV capsids remarkably augments targeted gene delivery to renal tubules with minimum off-target effects

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The development of effective methods for gene delivery to the kidney has long been sought. However, it still remains difficult to effectively transduce clinically relevant cell types in the kidney, such as renal tubular epithelial cells. To address such an unmet need in AAV vector-mediated gene delivery, AAV capsid engineering technologies have emerged and overcome some of the hurdles. For example, systemic intravenous (IV) administration of robust AAV capsids such as AAV9 and its derived mutants has shown promise in targeting the CNS, heart, skeletal muscle and other organs. The kidney, however, poses various challenges, making the approaches ineffective that would otherwise be effective in other organs. In this regard, we have recently reported that local renal vein (RV) injection of select AAV capsid mutants, but not other capsids or IV injection, can remarkably enhance renal transduction in mice. This augmentation effect is unique only to a subset of AAV capsid mutants and is not a shared attribute of the common AAV capsids, including AAV9. Here, we show that retrograde renal pelvis (RP) injection, a local route of administration (ROA) alternative to RV injection, can also remarkably enhance AAV vector-mediated renal gene transfer in mice by an order of magnitude when select AAV capsids are used. In this study, we injected mice via RP with a barcoded AAV library containing a total of 47 AAV capsids, and found that only 6 out of 47, including AAV-KP1, showed remarkable enhancement that is not observed when administered by IV. Subsequently, we tested AAV-KP1 as the prototype of these efficient capsids and compared it with AAV9 as the benchmark serotype. We found that the enhanced renal transduction of AAV-KP1 by RP injection is primarily attributed to augmented transduction in the proximal renal tubules, which are difficult to transduce with the common AAV capsids. To investigate the mechanism of proximal tubule transduction, we injected fluorescent microspheres by IV and RP and assessed the distribution of AAV vectors. Consequently, we found that, in contrast to mesangial accumulation following IV injection, RP injection resulted in efficient accumulation of microspheres in the cortical interstitial space. These results suggest that peritubular capillaries pose a significant barrier for transduction following IV injection, which can be overcome by RP injection, leading to efficient proximal tubule transduction from the basolateral side. In addition, by quantifying AAV vector genomes in the kidney and blood in mice shortly after AAV RP injection, we found that >10 times more vector genomes remained within the AAV-KP1 vector-injected kidney than in the AAV9 vector-injected kidney. Accordingly, blood vector concentrations of AAV-KP1 vector following RP injection were two orders of magnitude lower than those of AAV9, which substantially limits vector spillover to non-target organs, including the liver, by RP injection. On the other hand, off-target transduction of AAV9 was not different between IV and RP. Thus, RP injection of the select AAV capsids harbouring this peculiar biological attribute is a promising approach for effective and safe renal gene delivery.

OR53

Evaluation of cellular immune response to Adeno-associated virus-based gene therapy

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The number of approved or investigational late phase viral vector gene therapies (GTx) has been rapidly growing. The adeno-associated virus vector (AAV) technology continues to be the most used GTx platform of choice. The presence of pre-existing anti-AAV immunity has been firmly established and is broadly viewed as a potential deterrent for successful AAV transduction with a possibility of negative impact on clinical efficacy and a connection to adverse events.

Recommendations for the evaluation of humoral, including neutralizing and total antibody based, anti-AAV immune response have been presented elsewhere. This presentation, the output of the American Association for Pharmacutical Scientists (AAPS) working group on cellular immunity of AAV-based gene therapy, will cover considerations related to the assessment of anti-AAV cellular immune response, including review of correlations between humoral and cellular responses, potential value of cellular immunogenicity assessment, and commonly used analytical methodologies and parameters critical for monitoring assay performance. This manuscript was authored by a group of scientists involved in GTx development who represent several pharma and contract research organizations. It is our intent to provide recommendations and guidance to industry sponsors, academic laboratories, and regulatory agencies working on AAV-based GTx viral vector modalities with the goal of achieving a more consistent approach to anti-AAV cellular immune response assessment.

OR54

Effective treatment of mitochondrial complex III deficient mice with hepatocyte-targeted gene therapy

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Mitochondrial disorders comprise a large group of monogenic inborn errors of metabolism, typically with compromised respiratory chain (RC) function, leading to a wide spectrum of manifestations from myopathies to encephalopathies and multi-organ disease. Mutations in the RC complex III (CIII) assembly factor gene *Bcs1l* are the most common cause of CIII deficiency (1). The most severe of them is GRACILE (Growth Restriction, Aminoaciduria, Cholestasis, liver Iron overload, Lactic acidosis, and Early death) syndrome, described in Finland (2). Our group generated GRACILE mutation knock-in (Ser78Gly mutation in Bcs1l) mice that, in genetic backgrounds with or without a novel spontaneous mtDNA variant, recapitulate most of the clinical manifestations (3,

4). Complex III deficient *Bcs1l*$^{p.S78G}$ mice show a striking juvenile-onset progeroid disease with growth restriction, kyphosis, and decreased bone mineral density and fat mass – highly unusual manifestations for an OXPHOS disease. In line with this, they show widespread DNA damage response and senescence in the liver, kidney and exocrine pancreas, tissues that regenerate via cell cycle re-entry of differentiated parenchymal cells (5).

In GRACILE syndrome patients and Bcs1l mutant mice, early histopathology and CIII deficiency are mainly seen in the liver, and the concomitant systemic metabolic phenotypes (growth restriction, loss of adipose tissue, and hypoglycemia) are likely to be liver-dependent, as recently assessed (6).Therefore, to interrogate the systemic contribution of the metabolically compromised and senescent hepatocytes, we used AAVs, serotype 9 and hepatocyte-specific promoter, to express wild-type BCS1L in the *Bcs1l*$^{p.S78G}$ mice starting at presymptomatic age (P21).
In *Bcs1l*$^{p.S78G}$, *mt-Cyb*$^{p.D254N}$ juvenile mice, a single intraperitoneal injection of AAVs targeted the liver efficiently. After the injection, the assembly of the CIII is corrected and the functional changes like; DNA Damage, senescence, liver disease were partially of fully rectified. Moreover, the systemic phenotype like; body temperature, growth was completely or partially normalized, and the survival was doubled. We propose that the hepatocyte-targeted gene therapy in complex III deficient mice is an effective and novel strategy to treat mitochondrial progeria in an OXPHOS disease.

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OR55

Phenotypic correction of hematopoietic progenitors from Fanconi anemia group A knockout mice using non-viral gene therapy with *Sleeping Beauty* transposon vectors

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Lentiviral-mediated gene therapy of human $CD34^+$ hematopoietic stem cells (HSCs) derived from Fanconi anemia subtype A (FA-A) patients confers engraftment and proliferation advantage of phenotypically corrected HSCs in non-conditioned FA-A patients. We have previously demonstrated the applicability of the non-viral *Sleeping Beauty* (*SB*) transposon system for efficient and stable gene delivery in human $CD34^+$ cells. Here we address the feasibility of using this system for preclinical gene therapy in FA group A knockout (*Fanca*$^{-/-}$) mouse model. To optimize *SB* gene transfer into hard-to–transfect murine Lineage-negative (Lin^-) cells, wild-type (Ly.5.1) Lin^- cells were nucleofected with a minicircle (MC) *SB* transposon vector expressing the Venus fluorescent protein together with mRNA encoding the hyperactive SB100X transposase. Transfection efficiency at day 3 was >57% and increased to >70% $Venus^+$ cells 21 days post-nucleofection. $Venus^+$ cells maintained their ability to proliferate in CFU-C assays and efficiently engrafted primary and secondary recipient mice (Ly5.2). In the peripheral blood (PB), bone marrow (BM) and spleen (SPL) of primary and secondary recipients, $Venus^+$ cells were found in both myeloid and lymphoid lineages. Genome-wide insertion site profiling revealed a close-to-random distribution of *SB* transposon integrands.

Using the optimized nucleofection protocol, we next aimed to investigate whether gene therapy with the *SB* transposon system would be feasible in a *Fanca*$^{-/-}$ mouse model. Nucleofection of hTERT-immortalized skin fibroblasts from a FA-A patient (FA-52) with a transposon MC harboring the *GFP* and *FANCA* transgenes (MCT2GFASV40pA) together with mRNA encoding the hyperactive SB100X transposase resulted in a progressive increase in the frequency of GFP^+ cells from ~15% at day 2 post-nucleofection to ~50% after 56 days in culture. GFP^+ FA-52 clones were as resistant to the DNA damage-inducing compound mitomycin C (MMC) as HeLa cells, indicating successful phenotypic correction of the genetic deficiency of these cells. Western blot analysis confirmed FANCA expression in genetically corrected FA-52 fibroblasts after MMC treatment.

Following nucleofection of MCT2GFASV40pA together with the SB100X transposase in murine *Fanca*$^{-/-}$ Lin^- cells, approximately 20% of the cells maintained stable GFP expression for more than 26 days in liquid culture and corrected the MMC hypersensitivity. The average vector copy number (VCN) per diploid genome was 2.38. *In vivo*, the *SB*-mediated gene therapy of *Fanca*$^{-/-}$ Lin^- cells resulted in a progressive expansion of genetically corrected cells in 2 lethally irradiated *Fanca*$^{-/-}$ mice (from 1.1% and 7.8% GFP^+ cells at week 6 to 52.6% and respectively 34.7% GFP^+ cells in PB 18 weeks after transplantation). MMC treatment of primary recipient mice with <1% GFP^+ cells at week 6 after transplantation also resulted in a progressive multilineage reconstitution of genetically corrected cells reaching 77.3% GFP^+ cells 18 weeks after transplantation. Importantly, the very high repopulating ability of the genetically corrected HSCs was maintained in secondary recipient mice. These results demonstrate for the first time that the *SB* transposon system is a very promising non-viral alternative for genetic correction of HSCs that could be employed in future clinical gene therapy protocols for patients with FA and other inherited hematopoietic diseases.

OR56

Modulating binding affinity of aptamer-based loading constructs for efficient EV-mediated CRISPR/Cas9 delivery

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The CRISPR/Cas9 toolbox consists of modular nucleases that can be employed to efficiently modify genomic sequences with high specificity. Hence, these systems open new avenues in the development of gene-editing therapies. However, targeted delivery of the large Cas9-sgRNA ribonucleoprotein (RNP) complexes remains challenging due to immunogenicity, negative charge, and rapid degradation. An approach to potentially overcome most of these limitations is the use of extracellular vesicles (EVs) as intercellular delivery vehicles. EVs exhibit the natural ability to carry RNA and proteins across biological membranes and can be engineered to load biotherapeutic molecules and target specific tissues. To load Cas9-sgRNA complexes into EVs, sgRNAs containing MS2 aptamers and a fusion protein of CD63 and tandem MS2 coat proteins were expressed alongside Cas9 and VSV-G in HEK293T cells. To study the effect of binding affinity on Cas9-sgRNA delivery, both the interacting sgRNA MS2-hairpin and the RNA-binding domain of the MS2 coat protein were mutated. To separately study the effects of affinity on cargo loading and release, a UV-sensitive photocleavable protein (PhoCl) was included in the MS2-CD63 construct to maximize cargo release. We used a previously published fluorescent stoplight reporter system that can be activated by delivered Cas9-sgRNA complexes to measure functional Cas9-sgRNA delivery. We confirmed that adaptation of the sgRNAs did not adversely affect their functionality. Comparing Cas9-sgRNA delivery for the different modulated sgRNAs revealed that adapting binding affinity highly affects functional delivery (0.5% to 22.2%). A similar effect on functional delivery was seen after adaptation of the affinity of the RNA-binding domain of the MS2 coat protein. After UV-treatment, photocleavable MS2-PhoCl-CD63 fusion proteins revealed similar Cas9 delivery for most sgRNAs with varying affinities, indicating that cargo release, and not loading, was a limiting factor in aptamer-mediated Cas9 delivery. Here, we describe a novel way to optimize EV-mediated loading and delivery of Cas9-sgRNA complexes. Our results demonstrate that EVs are capable of functional Cas9-sgRNA complex delivery, and that modulation of binding affinity can be used to facilitate efficient Cas9 delivery.

OR57

A cell-targeted lipid nanoparticle technology with enhanced cell selectivity for *in vivo* DNA delivery

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Generation Bio aims to solve two key challenges to realize the full potential of genetic medicines, achieving non-viral redosable delivery of DNA cargos for prolonged gene expression and correction in the liver and unlocking delivery to extrahepatic tissues. To address these challenges, we have developed a cell-targeted lipid nanoparticle (LNP) delivery system that enables more selective

on-target delivery, leveraging compositional and LNP manufacturing process improvements to optimize particle characteristics.

In vivo delivery of DNA to the liver by use of non-viral delivery vehicles (*e.g.*, LNPs) has long been a challenge, predominantly owing to acute activation of DNA sensors largely present in immune cells. We hypothesized that improvements in selective biodistribution to hepatocytes would result in better tolerability and improved therapeutic index for LNP-delivered DNA. Owing to its size and rigidity as a double-stranded, helical molecule, DNA presents a unique challenge in manufacturing LNP particles that are small and uniform, characteristics critical for functional hepatocyte delivery. It has been shown that the helical, base-paired structure of DNA can be denatured through solubilization in alcohol to condense it into tightly packed spheroids, possibly *via* P-form DNA. We have developed an LNP manufacturing process that incorporates this approach to formulate closed-ended DNA (ceDNA), a linear, double-stranded DNA molecule with inverted terminal repeat (ITR) structures on the ends. The resulting LNPs were smaller and more monodisperse than those produced using traditional manufacturing processes. These LNPs manufactured by the new process showed an improved ceDNA liver/spleen biodistribution ratio in mice, were better tolerated, and resulted in higher transgene expression as compared to standard LNPs *in vivo*. LNPs manufactured using this new process with a ceDNA encoding coagulation Factor VIII (FVIII) demonstrated FVIII expression >100% of normal in the FVIII mouse model and the ability to redose in immunocompetent mice to boost expression.

We have also created unique LNP compositions that have reduced protein corona formation and prolonged blood residence time, which can be redirected to cells of interest using targeting ligands. Importantly, these stealth LNPs retain thei ability to undergo pH-driven endosomal fusion to deliver cargo to the cytoplasm of target cells. Using an N-acetyl-galactosamine (GalNAc) ligand as a well-validated hepatocyte targeting moiety, we have demonstrated ligand-dependent liver biodistribution and achieved *in vivo* expression levels comparable to non-stealth, traditional LNP compositions that rely on endogenous ApoE targeting.

Beyond GalNAc, we have also shown the ability to conjugate protein-based ligands to our stealth LNPs that show improved selectivity and offer the potential to reach additional cell types and receptors. As an example, we conjugated an scFv ligand with improved selectivity for the asialoglycoprotein receptor (ASGPR), as compared to other lectin receptors such as CD301. These LNPs demonstrate receptor binding, functional cell uptake in primary hepatocytes, and high levels of hepatocyte transgene expression when dosed intravenously in mice.

Work is ongoing to combine stealth LNP compositions and conjugation chemistry with unique targeting ligands to target non-hepatic cell populations *in vivo*.

OR58

Gene silencing in hematopoietic stem and progenitor cells with an apolipoprotein A1-based nanodelivery platform

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Immunotherapies targeting the adaptive immune system has revolutionized healthcare. Similarly, modulating the innate immune system holds great therapeutic potential but has been largely unexplored. Here, we present an approach involving small interfering RNA (siRNA) delivery to hematopoietic stem and progenitor cells in the bone marrow. To achieve this, we have developed a novel RNA delivery platform based on apolipoprotein A1 (ApoA1). ApoA1 acts as a structural stabilizer for lipid-based nanoparticles, and it has inherent affinity for myeloid cells and their progenitors. Using ApoA1, siRNA, and fatty molecules, we developed a library of myeloid cell-avid nanoparticles for siRNA delivery, called siRNA-aNPs. The library's individual nanoformulations were screened for physicochemical properties and their ability to induce functional gene silencing *in vitro*. From the library screening, we selected 6 representative compositions for *in vivo* evaluation. Using radiolabeled siRNA, we identified several siRNA-aNP formulations that preferentially accumulate in the bone marrow. Furthermore, using flow cytometry we demonstrated significantly higher uptake of our lead siRNA-aNP in hematopoietic stem and progenitors cells compared to standard lipid nanoparticles for siRNA delivery. In future experiments, we will evaluate the silencing efficacy of the lead candidate *in vivo*. Finally, we will set-up treatment studies in various disease models to show siRNA-aNPs' full therapeutic potential.

OR59

Skeletal muscle organoids for preclinical gene therapy with recombinant AAV vectors

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Duchenne Muscular Dystrophy (DMD) is an X-linked genetic disease caused by mutations in the *DMD* gene coding for dystrophin. Young DMD boys suffer from marked muscle degeneration which progresses into respiratory failure, cardiomyopathy and premature death. No curative treatment exists to date, but promising approaches are currently investigated in the clinic, such as gene therapy using vectors derived from the adeno-associated virus (AAV). In animal models of DMD, AAV gene therapy leads to long-term expression of therapeutic transgenes and to an almost complete phenotypic rescue. In patients, promising intermediate results have been obtained in clinical trials, however, these were sometimes accompanied by serious adverse events that were not anticipated in pre-clinical studies. Therefore, new experimental models are urgently required for the development of next generation AAV vectors, with an improved efficacy and safety profile.

In this context, human induced pluripotent stem cells (hiPSCs) derived from patient samples could provide a reliable alternative, as they can be differentiated into virtually any cell type in a high-throughput manner, and in more complex 3D organoids recapitulating the structure and function of the native tissues.

OR60

Human engineered skeletal muscles for advanced modelling of congenital muscular dystrophies and neuromuscular genetic therapies

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Skeletal muscle is a complex tissue composed of multinucleated myofibres supported by multiple cell types, compromised in severe incurable neuromuscular diseases such as muscular dystrophies. Lack of robust, human(ised) models is a major barrier towards development of novel neuromuscular therapies, including gene therapies. To tackle this challenge, we developed 3D human models of skeletal muscle tissue, enabling high-fidelity modelling of different morphological and functional defects in tissue compartments impaired in muscular dystrophies, with a focus on severe, early-onset forms known as congenital muscular dystrophies (CMDs). 3D muscle models were engineered from human immortalised myoblasts as well as from induced pluripotent stem cells (iPSCs) differentiated into myogenic, neural and vascular progenitor cells, and then combined within biomaterials to generate aligned myofibre scaffolds containing vascular networks and motoneurons. Engineered muscles recapitulated morphological and functional features of human skeletal muscle, providing a high-fidelity platform to study muscle pathology, such as emergence of dysmorphic nuclei in a severe CMD caused by mutant nuclear lamins (named *LMNA*-related CMD, L-CMD). To further validate this approach, we then tackled one the most common forms of CMD: those caused by mutations in genes encoding crucial extracellular matrix (ECM) components such as collagen type VI (ColVI). Despite the availability of mouse models and of primary cell cultures, this family of ColVI-related dystrophies, including the most severe Ullrich congenital muscular dystrophy (UCMD), remain poorly understood and incurable. Therefore, we developed a novel human muscle-ECM 3D model capable to reflect the spatio-temporal cellular environment of both human native tissues by combining aligned, striated myotubes derived from skeletal myogenic cells and ColVI matrix deposited by human fibroblasts. Remarkably, this new model recapitulated clinically-relevant UCMD phenotypic features, including muscle/joint contractures and compromised muscle contractility, which would have been otherwise not detected in standard 2D monolayer models. Finally, we will present recent data supporting the therapeutic relevance and potential of these new CMD *quasi-vivo* platforms, including testing two mutation-specific strategies based upon antisense-oligonucleotides and genome editors on newly-generated myogenic derivatives from patients with rare *LMNA* mutations (causing L-CMD), as well as common *COLIV* mutations (causing UCMD). Overall, this platform will open new avenues for downstream etiological and prognostic studies, whilst also facilitating development of genetic therapies for CMDs.

OR61

Hematopoietic organoids allow for the scalable generation of different immune cell subsets from induced pluripotent stem cells

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Cell-based immunotherapies represent a rapidly growing field and novel advanced therapy medicinal products (ATMPs) show highly promising efficacies in several (pre) clinical studies. The most prominent example are CAR-based anti-cancer immunotherapies, which rely on different effector immune cells such as T-cells, NK-cells or macrophages. Given the success of such therapeutic strategies and the increasing demand for highly effective immune cells using economic manufacturing pipelines, we here introduce a process that is streamlined for the generation of various immune cell subsets. To introduce an all-in-one solution, we have developed a platform, that (i) allows to produce different immune cells from human induced pluripotent stem cells (iPSC) and (ii) can be applied at different scales ranging from classical tissue culture plates to intermediate scale bioreactors, or even industry compatible bioreactors. This simple, scalable, and efficient method is based on the cultivation of hematopoietic organoid intermediates (hemanoids) in suspension culture, which can give rise to different immune cells depending on the cytokine cocktail used.

Irrespective of the immune cell type to be generated, early mesoderm priming of hiPSC resulted in the formation of hemogenic endothelium and subsequent emergence of early hematopoietic progenitors within the developing hemanoids. Following hematopoietic specification, continuous generation of either myeloid progenitors or more committed immune cells such as macrophages, DCs or NK cells can be directed by the addition of specific, lineage instructive cytokines and allows the weekly harvest of the cell product.

The addition of IL-3 and M-CSF to the hemanoids lead to the generation of iPSC-derived macrophages (iPSC-Mac) which displayed a highly pure CD45+/CD11b+/CD14+/CD163+ population, exhibited classical functionality and showed a transcriptional profile similar to blood-derived macrophages, though with a more primitive fingerprint. Of note, polarization with different cytokines induced pro- or anti-inflammatory phenotypes and modulated the response towards a secondary inflammatory stimulus. Using this knowledge, we have shown the superior functionality of standardized iPSC-Mac compared to state-of-the-art, monocyte-derived macrophages at different stages of drug development such as (i) safety/pyrogenicity testing in the Monocyte-Activation-Test (MAT) or (ii) testing of novel immune-modulatory drug candidates in potency assays.

The generation of CD45+/CD56+/CD3-/CD16- NK cells is perused following the addition of a lineage-instructive cocktail comprising IL-3, SCF, IL-15, IL-7 and FLT3L during hematopoietic specification and differentiation. Of note, iPSC-NK cells can be successfully purified and expanded

using genetically engineered K562 cells by more than 150x fold and showed efficient degranulation as well as cytotoxicity after contact with K562 cancer cells. When adding combinations of IL-3, IL-4, GM-CSF, SR1 and FLT3L to the hemanoids, we also demonstrate the production of CD141+/CLEC9A+/CD123+/CD33-iPSC-derived DCs resembling different DC subtypes. Compared to iPSC-Mac, iPSC-DCs show an increased potential to efficiently process antigens and a reduced phagocytotic activity.

In summary, we present a simple, scalable, and efficient method to generate different immune cells from human iPSC using a streamlined version for the all-in-one production, which paves the way for a highly attractive and economic pipeline to manufacture and/or evaluate next generation ATMPs.

OR62

Gene editing for Usher syndrome type 2A: unravelling photoreceptor degeneration mechanisms and exploring therapeutic potential using retinal organoids

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Usher syndrome is a prevalent and devastating autosomal recessive disorder characterized by sensorineural hearing loss and progressive vision loss. Mutations in the *USH2A* gene, encoding USHERIN, are the most common cause (85%) of Usher2, one of the most common inherited retinal diseases. Effective treatments for this condition are currently lacking as standard adeno-associated virus (AAVs) vectors gene augmentation therapy is not an option given the very large *USH2A* gene (15kb transcript).

Eye research has benefitted tremendously by landmark studies showing the potential of pluripotent stem cells to form retinal organoids to model diseases and to test treatments, such as gene therapy. Our study aimed to gain insights into the disease mechanisms underlying photoreceptor degeneration using retinal organoids derived from patients with a common pathogenic deep intronic (c.7595-2144A>G) variant and to demonstrate efficacy for gene editing AAV therapy.

We conducted a comprehensive molecular characterization using ScRNA sequencing demonstrating novel disease biomarkers. Interestingly, using a targeted Parallel Reaction Monitoring (PRM) proteome assay we observed a reduction in the essential USHERIN protein and other Usher2 complex-associated peptides in affected organoids, suggesting their involvement in disease pathogenesis. Additionally, validation experiments confirmed the contribution of disease phenotypes to the degenerative process.

Deep intronic mutations are amenable to gene editing, thus we designed a single AAV vector to deliver the genome editing machinery concentrating specifically on the c.7595-2144A>G mutation.

We treated Usher2a retinal organoids, specifically targeting photoreceptor cells, and assessed the cutting efficiency using various guide RNA (gRNA) combinations. Notably, we observed increased cutting efficiency with prolonged treatment duration. Furthermore, we explored the impact of cutting efficiency using different AAV capsid variants to deliver the effector nuclease. Our comparative analysis identified that a novel in-house developed capsid variant showed improved gene delivery efficacy in vitro in photoreceptor cells.

Our study offers promising insights on the complex mechanisms driving photoreceptor degeneration in Usher2A and into gene editing strategies for retinal therapeutic interventions. While further research is needed, our study highlights that the combination of stem cell technology and its derivative organoids, together with state-of-the art gene editing approaches offer a favorable therapeutic avenue to treat blindness.

OR63

Therapeutic efficacy of brain repopulation by hematopoietic-derived microglia-like cells in progranulin-deficient mice

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In humans, Progranulin (GRN) deficiency causes two lethal neurological diseases which currently have no cure. Bi-allelic loss-of-function (l-o-f) mutations in the *GRN* gene cause Neuronal Ceroid Lipofuscinosis type 11 (CLN11), a rare neuropathic lysosomal storage disease (LSD) manifesting between 13 and 25 years of age with cerebellar ataxia, retinitis pigmentosa, seizures, and cognitive decline. Mono-allelic GRN l-o-f mutations represent 5% of all cases of Frontotemporal dementia (FTD-GRN), the second most common form of dementia in people under 65 years of age (3-15/100.000 people aged 45-64). GRN-FTD presents with aphasia, progressive deterioration of language, social behavior, and independent living, ultimately leading to death between 3 and 15 years from disease onset. GRN is a secreted lysosomal protein that is constitutively expressed but particularly enriched in neurons, macrophages, and microglia.

Hematopoietic stem cell transplantation (HSCT) is the standard of care for several neurometabolic diseases. Additionally, the autologous transplantation of gene-modified hematopoietic stem and progenitor cells (HSPCs) showed significant benefits in clinical studies for severe leukodystrophies and neuropathic LSDs, representing a promising approach to address GRN deficiency. However, the slow-paced and modest repopulation of the central nervous system (CNS) by HSCT-derived microglia-like cells (MGL) still hinders GRN delivery to the CNS to promptly address the progressive neurological manifestations. Additionally, concerns arise regarding supraphysiological expression of GRN due to its pleiotropic, yet poorly defined functions, and its upregulation in several cancers.

Here, we show that the use of an optimized conditioning regimen that combines Busulfan and the CSF1R inhibitor PLX3397, which specifically depletes microglia/macrophages, results in near-complete replacement of microglia by MGL and partial correction of GRN deficiency in the CNS of *Grn*$^{-/-}$ mice upon wild type bone marrow transplant. GRN protein reconstitution in the CNS also normalized lipid metabolism in the brain of *Grn*$^{-/-}$ mice. To leverage the potential of HSCT to address GRN deficiency, we are combining our novel conditioning approach with autologous

transplantation of genome-edited HSPCs. To this end, we targeted GRN expression cassettes to safe harbor loci in human and mouse HSPCs. GRN-expressing HSPCs secreted functional GRN and maintained long-term multilineage differentiation capacity. The efficacy of genome-edited mouse and human HSPCs is currently being examined in immunocompetent and immunocompromised models of GRN deficiency.

Overall, our study provides unprecedented data on the efficacy of HSCT for the treatment of GRN deficiency when combined with an optimized, clinically relevant conditioning regimen based on Busulfan and PLX3397. These results bode well for the development of an autologous HSCT approach for CLN11 and FTD-GRN.

OR64

Development of an *ex vivo* hematopoietic stem cell gene therapy for frontotemporal dementia (FTD)

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Frontotemporal Dementia (FTD, OMIM:607485) is a severe and fatal adult neurodegenerative condition devoid of any cure or specific approved treatment. About 20% of familial FTD cases are caused by pathogenic loss-of-function mutations in the progranulin gene (GRN). GRN is a secreted lysosomal protein that functions as neurotrophic factor and regulator of neuroinflammation. Raising GRN levels in the brain of FTD patients, and in particular in microglia, may result in therapeutic benefit. Hematopoietic Stem Cell Gene Therapy (HSC-GT) based on the use of lentiviral vectors (LVs) for gene transfer offers the potential benefit of long-lasting delivery of robust GRN levels in the CNS of FTD patients, potentially restoring physiological microglia functions, modulating neuroinflammation and attenuating neuronal damage. We developed and tested two therapeutic LVs with a strong constitutive (human PGK) *versus* a novel inducible synthetic promoter (derived from HLA-DRA) able to safely deliver multiple copies of the human *GRN* cDNA in GRN knock-out (ko) cell models, inducing over-expression of the therapeutic protein which is then correctly secreted and taken up by target GRN knock-out cells. The new promoter was first validated *in vitro* and *in vivo* for specific and regulated expression in response to neuroinflammation. The two vectors were employed in the setting of a proof of concept of efficacy study of HSC-GT for FTD in $Grn^{-/-}$ mice where the gene corrected cells were administered intravenously or in the brain lateral ventricles. Evaluation of the mice treated in the on-going study has revealed that the overall approach is feasible and well tolerated, and capable of exerting beneficial effects modulating disease phenotypes. Well known soluble disease biomarkers (BMP and GluSph) in plasma, brain lipofuscinosis, microgliosis and lysosomal dysfunction were normalized in the treated *versus* control mice at 12 months after transplant. Cohorts of animals are still being evaluated to collect additional evidence supporting an HSC-GT approach and to define the best promoter and route for cell delivery.

OR65

Intrastriatal injection of S0112AAV2-GBA1 is an efficient strategy to treat patients suffering from Parkinson's disease related to *GBA1* mutations

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Coave Therapeutics is developing conjugated AAVs through the chemical conjugation of functionalizing ligands onto the AAV capsid. Compared to unconjugated AAVs, coAAV candidate S0112AAV2 displays enhanced distribution and transduction capacities following intrastriatal delivery in both rodents and non-human primates (NHPs), including robust transgene expression in basal ganglia. Through this approach, brain regions altered in Parkinson's disease (PD) can thus be readily targeted. *GBA1* mutations are the most important genetic risk factor for PD. Compared to idiopathic PD, PD-GBA1 patients present a younger age at onset, an accelerated disease progression and a shorter survival rate. The *GBA1* gene codes for the lysosomal enzyme β-glucocerebrosidase (GCase) that catalyses the breakdown of glucosylceramide (GlcCer). Restoration of GCase activity in PD-GBA1 patients is expected to normalize GlcCer degradation, and mitigate α-Synuclein aggregation and spread, thus slowing or halting disease progression. Using a pharmacological mouse model of reduced GCase activity, we showed that intrastriatal S0112AAV2-GBA1 injection leads to a dose-dependent restoration of GCase activity, preventing GlcCer and associated glucosylsphingosine accumulation in the striatum. In healthy NHPs, intrastriatal S0112AAV2-GBA1 administration was well tolerated with no reported safety issues. GCase activity and protein expression were substantially increased in many PD-related brain regions including the putamen, the globus pallidus and the substantia nigra. Altogether, our results demonstrate the biological advantage of coAAVs over unconjugated AAVs and illustrate the therapeutic interest of our approach for the treatment of PD-GBA1.

OR66

A new minimally invasive endovascular approach to the cerebello-pontine cistern enables improved AAV biodistribution in the central nervous system compared to cisterna magna injection

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Neurological disorders remain difficult to target because of limited access of therapeutic agents to central nervous system (CNS) structures, as the protective blood-brain barrier (BBB) significantly inhibits the efficacy of intravenous gene and drug delivery. Parenchymal brain injections or cerebrospinal fluid (CSF) delivery to the cisterna magna (CM) can bypass the BBB and improve delivery to target brain tissue, but carry an unfavorable risk profile. We developed a novel percutaneous transvenous technique, performed in the neuroangiography suite, to safely access

the CSF-filled cerebello-pontine angle (CPA) cistern located in front of the pons/brainstem. This approach is presently in clinical trials as a treatment for hydrocephalus and potentially resolves the challenge of safely delivering clinically useful gene and cell-based therapies to the brain in a reproducible fashion. Considering the characteristics and limitations of current techniques, here we compare biodistribution of scAAV9-CB-GFP following CPA or CM administration using an ovine model.

Eight sheep were allocated into CPA (n=4) and CM (n=4) cohorts. Lumbar puncture was performed into the lumbosacral intervertebral space (L7-S1). A 1.7F braided microcatheter with 0.014" micro-guidewire was introduced into the subarachnoid space and navigated into either the CPA or CM under fluoroscopic guidance. Cone-beam computed tomography fused with MR imaging was used to confirm accurate final placement of the catheter. scAAV9-CB-GFP (1.0×10^{14} vg in 2.7mL) was injected at 100µl/min. Animals were sacrificed 3 weeks post-procedure. Anti-GFP antibody immunohistochemistry was performed with (Abcam ab290). Vector genome biodistribution was determined by qPCR using primers and probes for the BGH poly (A).

CPA delivery of scAAV9-CB-GFP vector resulted in broad transduction of cells in the brain. Immunohistochemistry targeting GFP showed strong transduction in the motor and parietal cortices, thalamus, cingulate gyrus, gray matter adjacent to the corpus callosum, and cerebellum after CPA injection. This was comparable to the same regions infused through CM. Vector genome quantification of brain and spinal tissues demonstrated similar biodistribution between the two cohorts. CPA animals showed somewhat greater mean vector genomes in frontal cortex, parietal cortex, temporal cortex, and thalamus, although statistical significance was not achieved. Only modest biodistribution to peripheral liver tissue was observed with both injection routes, an order of magnitude lower than that observed in CNS tissues.

CPA delivery of AAV9 resulted in strong transduction of motor, parietal, temporal cortices, thalamus, cingulate gyrus, gray matter adjacent to corpus callosum and cerebellum. These results suggest that administration of gene therapy into the CPA is at least equivalent to CM and achieves diffuse distribution throughout the CNS. In light of the favorable safety profile observed with human use of the device, percutaneous endovascular injection into the CPA may provide a clinically safer, minimally invasive CNS access approach for delivery of gene and cell-based therapies that leverages established high-resolution imaging technology and a ubiquitous trained neurointerventional workforce. This percutaneous CNS access technology is applicable to other gene therapy treatments that require repeated administration (ex. oligonucleotide therapeutics).

OR67

Exploring prime editing as a novel approach for targeting diverse Fanconi anemia mutations

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Fanconi anemia (FA) is an inherited bone marrow failure syndrome characterized by impaired DNA damage response, resulting in increased chromosomal instability. Because of this deficiency, precise genome editing approaches to specifically correct disease-causing mutations may constitute promising therapeutic opportunities to correct hematopoietic stem and progenitor cells (HSPCs) from FA patients. In this context, prime editing (PE) emerges as a unique strategy that functions as a "search and replace" genome editing method, bypassing the need of generating double-strand breaks in the DNA and the use of a donor DNA template. To assess the applicability

of PE to efficiently target hematopoietic cells from FA patients, we explored the possibility of correcting the most frequent mutation found in FA patients from Spain (FANCA c.295C>T mutation). To this aim, we combined the use of a PEmax architecture and a prime editing guide RNA (pegRNA) in synergy with a nicking sgRNA (PE3 system). Next generation sequencing analysis confirmed that the PE3 system precisely corrected the c.295C>T mutation to the wild type sequence in up to 40% of FA-A lymphoblastic cells, demonstrating that PE efficiency is not compromised in FA cells. As expected, the corrected cells exhibited a marked proliferative advantage together with the reversion of the characteristic hypersensitivity of FA cells to mitomycin C, an interstrand crosslinking agent . Importantly, different pegRNAs have been developed to correct other frequent mutations reported in different FA genes, including the most common mutation worldwide: the c.3788_3790delTCT *FANCA* mutation, as well as c.67delG *FANCC* mutation. Our results show that PE efficiency depends on the specific targeted genomic region, although reveal the potential for designing novel pegRNAs to correct the a significant number of FA mutations.

Moving to our target population, the HSPCs, the use of optimized in vitro transcribed PEmax allowed us to reach on target editing rates up to 28.0% and 30.1% in cord blood (CB) and mobilized peripheral blood (mPB) CD34+ cells from healthy donors, respectively. In all cases the generation of indels in the targeted region was minimal (<1%). By means of the cell sorting of different HSPC populations and the analysis of engrafted cells in immunodeficient transplanted mice, we also demonstrated that PE efficiently targets the most primitive human HSPCs. Overall, our results demonstrate, for the first time, the feasibility of using PE as a precise genome editing tool to target different mutations in FA and efficiently edit LT-HSPC from healthy donors, ssuggesting that PE might constitute a next generation gene therapy approach for personalized medicine in FA patients.

OR68

Inflammation and cellular senescence are uncharted barriers to efficient gene editing of human hematopoietic stem cells and T cells

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Gene editing (GE) in hematopoietic stem and progenitor cells (HSPCs) and T cells represents a revolutionary intervention for the treatment of a plethora of hematological conditions. Despite rapid technological advances based on GE technologies, many hurdles must be faced to improve GE efficiency and ameliorate HSPC repopulating potential upon ex vivo engineering. We previously showed that the concomitant exposure of nuclease-induced Double Strand Break with DNA repair template for Homology Directed Repair (HDR) delivered by AAV6 triggered the activation of the p53-mediated DNA Damage Response (DDR) pathway constraining HSPC proliferation and yield, suggesting that DDR-related cellular programs may inadvertently contribute to HSPC dysfunction upon GE. Protracted DDR signaling 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 the activation of inflammatory programs. Yet, whether GE could have durable and long-term consequences on the functionality of HSPCs remains to be elucidated. By integrating

transcriptional analysis (up to the single cell level) with innovative imaging-based cellular assays, we reported accumulation of senescence markers p16 and Senescence-Associated β-Galactosidase and pro-inflammatory programs induction across ex vivo engineered HSPC subsets from cord blood (CB) and mobilized peripheral blood (mPB) and in vivo in the human graft. Consistently, we found open chromatin at promoters of senescence-gene categories 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. Similar data were also observed in ex vivo activated and genetically engineered T cells. Mechanistically, we observed an ATM-p53-dependent activation of inflammatory cytokines in edited HSPCs. In this context, temporary inhibition of p53, IL1 and NF-kB pathways at the time of GE resulted in reduced senescence burden, increased clonogenicity of edited HSPCs ex vivo and ameliorated long-term hematopoietic reconstitution in xenotransplanted mice. In vivo clonal tracking of HDR-edited HSPCs by a recently developed barcoding-based strategy revealed that mitigation of senescence responses improved polyclonal reconstitution of individual edited HSPCs. Finally, by whole exome sequencing (WES) we reported no signs of increased genotoxicity upon transient senescence modulation or anti-inflammatory strategies. Our findings define senescence and inflammatory programs as barriers to CRISPR-Cas9 efficient engineering and pave the way for the development of novel strategies based on senescence modulation and anti-inflammatory molecules for future gene therapy applications.

OR69

Base editors provoke non-predictable chromosomal translocations and off-target editing as uncovered by CAST-Seq

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Base editing has emerged as a promising alternative to designer nuclease-based genome editing, in part, because genetic alterations can be achieved without introducing DNA double-strand breaks (DSBs). Despite the promise of reduced genotoxicity, a comprehensive analysis of base editing prompted genomic rearrangements has been missing. To this end, we employed CAST-Seq to identify gross chromosomal aberrations and off-target (OT) activity in primary human T cells that were edited side-by-side with either CRISPR-Cas9 nuclease, the adenine base editors ABE8e, one of three different cytosine base editors (evoCDA1-BE4max, TadCBEd, CBE-T1.52), or Cas9-D10A nickase. Using multiplexed amplicon sequencing (rhAmpSeq), we determined editing frequencies at three genomic target loci, *CCR5*, *EMX1*, and *FANCF*, and at the identified OT sites. Upon nucleofection of T cells, on-target editing reached 70-78% for the nucleases and 44-84% for the base editors. In contrast, in T cells edited with Cas9-D10A nickases, the root of the four base editors employed here, structural variations at target and OT sites remained close to background levels. When applying CAST-Seq in CRISPR-Cas9 edited T cells, we identified OT sites previously reported for these nucleases. Furthermore, we detected extensive chromosomal rearrangements at the target loci, stretching 15-30 kb around the respective cleavage sites, and uncovered 10

hitherto unreported OT-mediated translocations. Conversely, while ABE8e expression incited only few chromosomal aberrations, editing at 17 of the more than 40 identified OT sites surpassed 10%. Among the CBEs tested, evoCDA1-BE4max triggered the highest amount of OT effects. Notably, the total number of structural variations in evoCDA1-BE4max edited cells exceeded those found in Cas9 nuclease edited T cells by ~3-fold. Likewise, rhAmpSeq revealed extensive C to T conversion (up to 60% of alleles) as well as mutagenesis (up to 7% of alleles) at the nominated OT sites. In contrast, T cells edited with the recently developed TadCBEd or CBE-T1.52 showed only marginal on-target aberrations (up to 6% of alleles) or OT activity (<1% of alleles). In conclusion, our study reports for the first time the genome-wide detection of chromosomal rearrangements and OT effects prompted by base editors. While OT effects in TadCBEd and CBE-T1.52 edited T cells were in an acceptable range, our data uncovered the high genotoxic potential of ABE8e and in particular of the hyperactive evoCDA1-BE4max. These two base editors provoked mutagenic OT editing at sites that were not altered by Cas9 nucleases complexed with the same gRNA. This implies that the mechanism of OT activity of base editors is different from that of CRISPR-Cas nucleases, and that nucleases cannot be used as a surrogate for identifying base editor sparked OT effects.

OR70

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, at best, 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 dependent on the type of dystrophin mutation, 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. We have thus 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 various microRNAs (miR) binding its 3' untranslated region (3'UTR). Using a Cas9/gRNA ribonucleoprotein (RNP) complex we systematically disrupted the binding sites of such miRs at the DNA level in human DMD and murine myoblasts to permanently upregulate utrophin. Among the different candidates, Let-7c miR binding site (BS) appeared to be crucial for UTRN repression and its disruption induced a 3.5- and 3-fold increase of UTRN expression at the mRNA and protein levels

respectively. Interestingly, Cas9/gRNA mediated disruption was as efficient as the complete removal of Let-7c BS in upregulating UTRN expression. Same results were obtained in three-dimensional human DMD cultures, where let7c 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 Let7c targeting gRNA. Finally, we evaluated this strategy in the *mdx* mouse model of Duchenne by systemic 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). Overall, these findings provide the foundations for a universal gene editing therapeutic strategy for DMD.

OR71

Stem cell based gene therapy for recombinase deficient-SCID

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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 stem cell donor, but developing such therapy for RAG1/2 has proven challenging, given the high expression levels needed, especially for RAG1.

We tested clinically relevant lentiviral SIN vectors with 8 different internal promoters driving codon optimized versions of the *RAG1* or *RAG2* genes to ensure optimal expression. We used *Rag1-/-* or Rag2-/-mice as a preclinical model for RAG-SCID to assess the efficacy of the various vectors at low vector copy number. In parallel, the-conditioning regimen in these mice was optimized using busulfan instead of commonly used total body irradiation. 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.

A clinical trial for RAG1-SCID (RECOMB) has been initiated, while for RAG2 a clinical batch vector has been generated in preparation for a Phase/II trail in 2024. Two patients have thus far been included in the RAG1-SCID trial with a favourable clinical and immunological outcome, and polyclonal haematopoiesis. The RECOMB trial has a unique multinational design with clinical sites in Europe, Asia and Australia. Patients' mobilized stem cells will be sent to Leiden with, genetically modified and after QC returned to these centres as cryopreserved IMP. Thus, the paradigm of this consortium (cells travel, while patients stay home) has become realistic and may serve as a prototype for other gene therapy trials for rare diseases.

OR72

A groundbreaking IL-1RA-based gene therapy platform for treating a spectrum of inflammatory diseases

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The IL-1 pathway plays a crucial role in safeguarding the body against infections, but its dysregulation can cause chronic inflammation implicated in various autoinflammatory disorders. These IL-1 diseases affect diverse patients with genetic predispositions or multifactorial risk factors. These conditions can present with symptoms such as recurrent fever, skin rashes, joint pain, systemic inflammation, and other associated disorders. The severity and specificity of symptoms can differ depending on the disease and the patient's unique conditions. Despite the availability of biological treatments, these therapies only yield satisfactory outcomes for some patients, leaving many individuals unresponsive to these interventions. The complexity of IL-1-related diseases, their diverse underlying mechanisms, and the heterogeneity of patient responses contribute to the challenges in achieving universal efficacy with existing treatments. Therefore, there is a pressing demand for novel therapeutic approaches that can address the unmet needs of refractory patients and improve overall treatment outcomes. Our research endeavors led to the development of an innovative therapeutic approach targeting IL-1-mediated inflammation. Leveraging the inherent ability of immune cells to migrate across different tissues, we harnessed their potential to transport and release the anti-inflammatory cytokine IL-1 receptor antagonist (IL-1RA) specifically to inflamed tissues. We devised an innovative approach using lentivirus (LV)-mediated gene transfer and autologous hematopoietic stem/progenitor cells (HSPCs) as a platform for delivering the IL-1RA. The transplantation of IL-1RA-transduced HSPCs showed remarkable efficacy in counteracting acute neutrophil recruitment in chimeric mice subjected to an inflammatory stimulus mimics gouty arthritis. Moreover, IL-1RA gene therapy reduced the clinical manifestations of Cryopyrin-associated periodic syndrome, including weight loss, leukocytosis, serum IL-6 production, and inflammation across multiple tissues. In the experimental autoimmune encephalomyelitis model of multiple sclerosis, IL-1RA gene therapy prevented disease-related mortality and alleviated the severity of symptoms. In conclusion, our study showcases the groundbreaking potential of the IL-1RA gene therapy platform as a definitive treatment for a wide range of inflammatory disorders. By specifically targeting IL-1-mediated inflammation and delivering IL-1RA to inflamed tissues, our innovative approach offers long-lasting relief and represents a significant breakthrough in the field. This gene therapy promises to provide durable remission and improve the overall quality of life for individuals affected by these debilitating conditions.

OR73

Base editing restore cellular phenotype of T cells of patients with Hyper-IgE-Syndrome

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Hyper-IgE-syndrome (HIES) is a rare immunodeficiency characterized by recurrent skin and pulmonary abscesses, elevated IgE serum levels and a deteriorating quality of life in general. Disease-causing mutations in HIES patients are mainly found in the regions of the *STAT3* locus that encode the DNA-binding or the SH2 dimerization domain. *STAT3* is a tightly regulated gene expressed in two isoforms: STAT3 alpha, which promotes proliferation and STAT3 beta, which suppresses cell growth. Hence, a continuously controlled balance between the two isoforms is crucial. The mutations affecting DNA binding or dimerization of STAT3 interfere with its function as a transcription factor and impede the activation of downstream target gene expression, such as *SOCS3*. As a consequence, in HIES patients, naïve T cells cannot differentiate into Th17 cells, rendering them highly susceptible to infections. However, an allogeneic hematopoietic stem cell transplantation is not indicated because the disease burden does not outweigh the risks associated with conditioning and graft-versus-host disease. In this study, we explored the feasibility of correcting the *STAT3* gene in patient T cells harbouring mutations K340E or R382W. Both mutations are located in the DNA binding domain, thus preventing downstream target gene activation after phosphorylation of STAT3. We designed a cytosine base editor (CBE) to correct the K340E allele and an adenine base editor (ABE) for R382W in order to convert the underlying point mutations back to wild type. Upon optimization of gRNA design and mRNA transfer to patient T cells, up to 98% and 86% of K340E and R382W alleles, respectively, were edited. Based on NGS analysis, our gene editing approach restored wild type STAT3 expression in 90% or 68% of patient cells. An mRNA expression analysis revealed a ~4:1 ratio between the STAT3 alpha and beta isoforms in the base edited cells, comparable to that found in healthy donor T cells. Finally, we verified functional rescue of the patient T cells by evaluating STAT3-mediated activation of a downstream target gene. Upon stimulation of base edited T cells with IL-21, STAT3 was phosphorylated and *SOCS3* expression upregulated some 30-fold, thus to similar levels observed in control T cells. In conclusion, our proof-of-principal study demonstrates the feasibility of using base editing in HIES patient T cells to restore physiological expression of functional STAT3.

OR74

Base-editing as a safe and highly effective alternative treatment for X-SCID compared to CRISPR-Cas9 nuclease editing with an AAV donor

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Current *ex vivo* lentivector hematopoietic stem/progenitor cell (HSPC) gene therapy for the treatment of X-linked Severe Combined Immunodeficiency (SCID-X1) provides substantial clinical benefit, but there is a risk of insertional oncogenesis. Genome editing holds great potential for improving safety and efficacy in this context. The CRISPR/Cas9 nuclease/AAV-*IL2RG* system creates a double-strand DNA break (DSB) at the target site, followed by homology-directed repair (HDR) to achieve a targeted insertion (TI) of *IL2RG* cDNA, addressing most of the IL2RG mutations. However, DSBs induce DNA damage responses (DDR) that may be compounded by adeno-associated virus (AAV) donors, potentially affecting HSPC fitness and long-term engraftment.

In this study, we compare the safety and efficacy of genome editing approaches to correct the *IL2RG*c.444C>T mutation in HSPCs from a SCID-X1 patient. We utilize CRISPR/Cas9/AAV-*IL2RG* genome editing (GE) and base editing (BE) using an Adenine Base Editor (ABE8e-SpCas9) that avoids DSBs and exposure to viruses. To demonstrate restoration of *IL2RG* function, GE- and BE-HSPCs from the SCID-X1 patient were transplanted into immunodeficient NOD.Cg-Prkdcscid Il2rgtm1Wjl Tg mice pups (NSGS). Transplanted animals were harvested at 16-24 weeks post-transplantation. We observed similar engraftment rates in both cohorts (GE: 13.01% ± 7.81, BE: 13.52% ± 7.27, naïve: 10.17% ± 10.67, HD: 15.90% ± 9.46).

IL2RG is essential for the differentiation and function of lymphocytes, accounting for the profound cellular and humoral immune deficiency in SCID-X1 patients. T cell reconstitution in transplanted mice was evaluated by flow cytometric analysis for the percentages of circulating T cells (CD3+) and IL2RG encoded γc expression at week 16. Although there was no significant difference in the percentage of CD3+ cells between GE (79.77% ± 35.65) and BE (91.45% ± 22.39) HSPC-transplanted mice, IL2RG expression in CD3+ T cells was significantly ($p=<0.0001$) higher in BE mice (93.85% ± 4.30) compared to GE mice (59.89% ± 21.01).

Molecular analysis for the genetic correction of SCID-X1 HSPCs was assessed by digital droplet PCR for TI in GE HSPCs and high-throughput sequencing of the target site for BE HSPCs, respectively. Mice bone marrow sorted for hCD45 cells revealed a significantly higher rate of mutation repair achieved by BE (84.55% ± 19.14) than GE-mediated rates of TI (55.36 ± 18.01). Whole exome sequencing and high-throughput sequencing of potential off-targets, nominated by in vitro CHANGE-seq assay, confirmed the highly specific targeting of both genome editing strategies. However, ultra-deep whole-genome optical mapping revealed complex on-target insertions and chromosome foldbacks following GE, the latter of which became undetectable after a further 2-week culture. No genomic aberrations were detected following BE.

In conclusion, BE-SCID-X1 HSPCs displayed comparable engraftment, higher γc expression, and significantly higher levels of genetic correction in transplanted mice compared to GE-SCID-X1

HSPCs. However, the advantages of the BE-HSPC approach, which achieves greater efficiency with no detectable chromosomal structural variations (SVs), are balanced by the substantial regulatory burden of implementing each mutation. In contrast, GE potentially offers a "universal" approach of GE TI but is less efficient and carries risks of SVs that appear to be short-lived.

OR75

Establishment of a genome-wide CRISPR/Cas9 screening method for the identification of cellular factors that affect AAV production

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The development of gene therapy pharmaceuticals offers a great opportunity to treat or even permanently cure some previously untreatable diseases. Currently the most promising gene delivery systems for in vivo gene therapies are recombinant adeno-associated virus (rAAV) vectors, which show great potential due to their lack of pathogenicity, low immunogenicity and the ability to mediate long-term, episomal expression. Despite these benefits, the available production systems have limitations in the large-scale production of rAAV vectors.

Depending on the indication, $1x10^{14}$ viral genomes (vg) per kg body weight or more are required and there is an increasing trend towards the use of gene therapy approaches for the treatment of common diseases. As a result, the current titers of typically up to $1x10^{14}$ vg/l achieved by transient rAAV production are insufficient to meet the future demands.

The high variability of transient transfection, the poor scalability of adherent production systems and the suboptimal yield in full vs. empty viral particles during production (i.e. the infectious titer), all contribute to the limitations of the conventional rAAV production systems.

In order to overcome this production gap, we developed and implemented a screening method with the aim to identify cellular targets supporting or repressing AAV production in a scalable AAV suspension packaging cell line. To this end, we created a novel genome-wide Lentivirus-AAV-Cas9 screening library (LAC library) containing approximately 72,000 different sgRNAs for 18,000 human genes to identify factors that positively or negatively affect rAAV production. By analyzing rAAV particles using next-generation sequencing, we identified and quantified thousands of different barcodes from our library, several of which were strongly upregulated or downregulated due to CRISPR-mediated gene inactivation, making them interesting cellular targets to support improved rAAV productivity. These identified cellular factors are currently being further verified and characterised for their impact on rAAV production by various methods.

The presented results therefore describe a novel screening approach that supports the generation of optimised rAAV production cell lines, with improved productivity and scalability. An important aspect of this work is the validation of the screening method itself, which could potentially be applied to other cell lines and viruses as well.

OR76

Advancing the manufacture of hiPSC-derived cardiomyocytes in bioreactors through WnT activation and dissolved oxygen control

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Myocardial infarction causes a significant loss of cardiomyocytes and due to the limited capacity of the adult human heart to regenerate, heart function continuously deteriorates, leading to heart failure. Advances in cell-based therapies, such as injection of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs), have shown potential in improving cardiac regeneration. However, generating a therapeutic number of cardiomyocytes (> 1 billion hiPSC-CMs per patient) is extremely laborious and time-consuming, hampering their clinical application. Recently, it was demonstrated that GSK-3β pathway inhibition, and removal of cell-cell contact supress cardiac maturation and enable expansion of hiPSC-CMs in conventional static culture systems. In this work, we adapted this method to a bioreactor-based protocol aiming to improve process scalability, reproducibility and to maximize hiPSC-CM expansion factor and cells' quality attributes.

hiPSC-CMs were cultured as 3D aggregates in 200mL stirred-tank bioreactors (STB). CM expansion was induced by CHIR99021 addition through continuous perfusion mode, and aiming at bioprocess intensification we modulated the dissolved oxygen (DO) throughout the process. When DO was controlled at low levels, i.e. mild-hypoxia (10% O_2), a reduction of 30% in reactive oxygen species production together with an increase of 70% in the concentration of proliferative cells ($Ki67^+$ cells) was observed when compared to the hiPSC-CMs that were cultured in STB operated under atmospheric normoxia conditions (STB-21% O_2). In fact, after 11 days of culture, a higher CM expansion factor was attained in STB-10%O_2 (10.3), relative to the values achieved in the STB-21%O_2 (4.4) and static culture system (3.6), yielding approximately 400 million hiPSC-CMs per 200mL bioreactor run. Importantly, our results also demonstrated that STB culture improved hiPSC-CMs purity as higher percentages of cells expressing cTnT and α-actinin were attained (>98%) when compared to the static culture (90%). In addition, hiPSC-CMs aggregates showed spontaneous beating, immature cardiac phenotype (confirmed by confocal immunofluorescence microscoscopy and RNA-seq analysis) and were able to further mature in culture, displaying improved CM maturation features at the level of gene expression, microstructure (increased sarcomere alignment) and function (increased cardiac action potential).

We then scaled-up the optimized CM expansion bioprocess by 10x to ensure the production of clinically relevant numbers of functional hiPSC-CMs (> 1 billion hiPSC-CMs), by validating the protocol optimized in 200mL STB in 2L scale (UNIVESSEL SU, Sartorius). A comprehensive approach focusing on the engineering characterization of the 200mL STB was established and several scaling criteria were compared to determine the most appropriate approach. We determined the impeller power number (Np), Power input per volume (P/V), and the mixing and suspension dynamics of the 200mL STB. Scaling-up validation was then performed by keeping constant P/V between the different systems.

In conclusion, in this work we developed a scalable bioprocess for the production of hiPSC-CMs in STB, showing the importance of controlling the DO at low levels to improve hiPSC-CMs expansion factors and their quality attributes.

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OR77

Development of an ex-vivo precision gene engineered B cell medicine platform and demonstration of engraftment without pre-conditioning in non-human primates

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Ex vivo gene and cell therapies are transformative medicines which can treat previously intractable diseases. However, there are barriers to the broad adoption of these efficacious therapies as current platforms lack high biosynthetic capacity, durability, and cannot be administered as an off-the-shelf medicine without preconditioning to facilitate engraftment. Terminally differentiated human plasma cells (PCs) derived from genetically engineered B cells (termed B Cell Medicines, BeCMs), potentially offer natural longevity (persisting for decades), capacity for high levels of protein secretion (thousands of Ig molecules/cell/sec), and the ability to engraft without host preconditioning. BeCMs are produced via CRISPR/Cas9-based HDR mediated transgene insertion followed by *ex vivo* differentiation into plasma cells. Focusing on engineering at the CCR5 safer harbor site, our platform achieves gene knockouts with greater than 90% efficiency as well as targeted HDR-mediated gene insertions at frequencies as high as 60% without selection. A BeCM prototype engineered to express firefly luciferase was produced and differentiated into antibody secreting cells (ASC) with a phenotype of >90% CD27+CD38+ cells. The BeCM prototype was injected via IV into immunodeficient mice, with rapid (< 3 days) bone-marrow-homing and durable engraftment (>100 days). To further illustrate the modularity of the BeCM platform we engineered B cells to produce either lysosomal storage disease (LSD) enzyme acid sphingomyelinase (ASM), clotting factor IX (FIX), or an anti-CD19/CD3 bispecific T cell engager, anti-CD19/CD3 scFv. These examples demonstrated that BeCMs produce proteins with enzyme specific activity much higher than standard recombinant proteins (ASM), are stably expressed for over 4 months *in vivo* (FIX), and show efficacy in tumor treatment (anti-CD19/CD3 scFv). A key step in developing BeCMs involves preclinical testing in a model with an intact immune system similar to that of humans. We developed an *ex vivo* method to engineer, expand, and differentiate non-human primate (NHP) peripheral blood B lymphocytes into PCs. We utilized zirconium-89-oxine cell radiolabeling for high sensitivity PET/CT tracking of *ex vivo* expanded and differentiated autologous PCs in unconditioned NHPs. We demonstrate that *ex vivo* expanded and differentiated NHP PCs rapidly home to and engraft in the bone marrow, liver, and spleen of unconditioned, immune competent hosts. Engraftment without preconditioning broadens BeCM clinical utility for patients where preconditioning toxicities are unacceptable or outweigh therapeutic benefit and could facilitate additional rounds of treatment as needed. BeCMs capable of expressing therapeutically relevant

transgenes have the potential for broad and meaningful therapeutic utility in rare diseases, cancer, and beyond.

OR78

Adenine-base editing corrects the most common *ABCA4* mutation causing Stargardt disease

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Stargardt disease is an inherited neurodegenerative disease that leads to macular degeneration and blindness caused by loss-of-function mutations in the *ABCA4* gene. Currently, there is no approved therapy. In this study, we developed a dual adeno-associated virus (AAV) split-intein adenine base editing approach to correct the most common mutation in *ABCA4* (c.5882G>A, p.G1961E).

To test different split-intein- and base editor versions, we used mutation-carrying 293T cells. To assay base editing in relevant target cells (photoreceptors and retinal pigment epithelial (RPE) cells), we developed an engineered human retinal organoid model and generated a humanized mouse model carrying the *ABCA4* c.5882G>A mutation. Furthermore, using the adjacent adenine (c.5883A, *wobble base*), we tested the same base editing strategy on wild-type human retinal explants and in RPE/choroidal explants. Finally, we subretinally injected six adult Cynomolgus macaques and assayed base editing rates at the genomic DNA level, at the RNA level and on the DNA level from sorted rod and cone photoreceptors.

Base editing on the Stargardt target base (c.5882G>A) and the adjacent wobble base (c.5883A) were similar in lentivirus-carrying HEK293T cells and in human retinal organoids. Therefore, the wobble base assay can be used to assess editing rates in wild-type models (human explants and NHPs). Our split-intein adenine base editor AAV vector led to 5-15% editing in retinal organoids and 10-20% editing in human retinal explants, assayed at the genomic DNA level. Next, we injected the optimized vector construct into mutation-carrying mice and achieved high *in vivo* gene correction, where ~50% of photoreceptors and ~80% of RPE cells showed editing. In injected NHPs, base editing rates were found to be ~40% in cones, ~28% in rods and up to ~99% in RPE cells.

We developed an optimized vector candidate for the correction of the *ABCA4* c.5882G>A mutation in human models (retinal organoids, human explants). Using this optimized vector, we achieved high base editing rates after in vivo injection in mice and NHPs. These results demonstrate that AAV-mediated base editing can lead to a precise genetic change in the nervous tissue in non-human primates.

OR79

Prime editing precisely corrects prevalent pathogenic mutations observed in glycogen storage disease type 1b (GSD1b) patients

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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, including hepatic glycogenolysis. Patients with mutations in this gene exhibit multiple severe clinical manifestations including severe 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. Correcting SLC37A4 gene mutations in fewer than 10% of hepatocytes may be sufficient to reverse many manifestations of this disease.

Prime Editing (PE) 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), with minimal byproducts at the edit site, minimal off-target activity and minimal risk of large chromosomal alterations or genotoxicity sometimes observed with CRISPR-Cas9. We developed a lipid nanoparticle (LNP) delivery system to deliver Prime Editing drug components to the liver by a single intravenous infusion. The Prime Editor can be delivered as an engineered mRNA combined with engineered Prime Editor guide RNA (pegRNA). Our therapeutic approach utilizes LNP-mediated delivery of these components to the liver to correct the prevalent SLC37A4 mutations, p.G339C and/or p.L348fs, which are observed in ~50% of patients.

Comprehensive high-throughput screening for Prime Editors led to the identification of initial hits that precisely correct either the SLC37A4 p.G339C or p.L348fs mutations. Initial lead performance was assessed in primary hepatocytes isolated from humanized mice in which the mouse SLC37A4 gene was replaced with the human gene harboring either the G339C or L348fs mutations or in patient iPSC-derived hepatocytes. LNP-mediated delivery resulted in editing efficiencies up to 80% *in vitro*. A similar assessment of performance was performed *in vivo* following single intravenous delivery of LNP formulated PE candidates. Seven days post dosing, precise genomic correction of the G339C and L348fs mutations was observed in whole liver (total liver alleles) at rates up to 43% in vivo. These edits resulted in high levels of correct SLC37A4 transcripts.

These results demonstrate LNP can effectively deliver an all-RNA Prime Editing cargo to the liver at high efficiency and show that Prime Editing can efficiently and precisely correct pathogenic mutations causing GSD1b at efficiencies in excess of those believed to be required to reverse manifestations of disease. Updated results will be provided at the time of the meeting.

OR80

EMD-301 - a potent “one and done” gene editing-based therapy for hypercholesterolemia-related disorders up regulates LDLR expression and boosts LDL-C uptake

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Hypercholesterolemia, a condition characterized by consistently high levels of low-density lipoprotein cholesterol (LDL-C) in the blood, is a leading cause of Atherosclerotic Cardiovascular Disease (ASCVD), and has a significant impact on global health, contributing to millions of deaths each year. Even when used in combination, the current lipid-lowering therapies for hypercholesterolemia, such as statins and PCSK9-inhibitors, fall short in achieving desired LDL-C levels in approximately 7 million patients, and require lifelong management. Here we present EMD-301, a potentially “one-and-done” in-vivo therapeutic strategy for direct and long-lasting upregulation of LDL receptor (LDLR) expression, resulting in increased LDL-C uptake. EMD-301 is a lipid nanoparticle (LNP)-based composition carrying the guide RNA and mRNA of a unique CAS nuclease, that has been successfully engineered to enhance its activity and specificity. By targeting and truncating regulatory elements in the 3’ untranslated region (3’UTR) of the LDLR gene, EMD-301 stabilizes LDLR mRNA, leading to elevated LDLR expression. Preliminary in-vivo experiments conducted in mice demonstrated that truncation of the LDLR 3’UTR in approximately 20% of hepatocytes resulted in a remarkable reduction of over 60% in LDL-C blood levels without any adverse effect on plasma triglyceride levels. In human cells, gene editing with EMD-301 resulted in over a 2-fold upregulation of LDLR mRNA levels and a 4-fold increase in membrane-bound LDLR, significantly enhancing LDL-C uptake by 3-4-fold. Importantly, this innovative therapeutic strategy did not involve any detectable off-target effects. Furthermore, a comparative analysis demonstrated that EMD-301, as a monotherapy, significantly outperformed a combination treatment of PCSK9 knockout and statins in terms of increasing LDL-C uptake. These findings highlight the immense potential of our in-vivo gene-editing approach as a safe, effective, and durable therapy for hypercholesterolemia, irrespective of its underlying genetic factors.

OR81

AAV9-Mediated gene therapy in a knock-in mouse model of infantile neuroaxonal dystrophy

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Infantile neuroaxonal dystrophy (INAD) is a rare and lethal paediatric neurodegenerative disease. It is caused by biallelic mutations in the *PLA2G6* gene, which codes for the enzyme calcium-independent phospholipase A2. Patients present with progressive neurological symptoms between six months and three years of age, with mortality typically occurring by 10 years old. No disease modifying treatments are available. We conducted an in-depth characterization of

the *pla2g6-inad* knock-in mouse model. Following characterization, we investigated the therapeutic potential of an AAV9.h*PLA2G6* vector administered intracerebroventricularly to neonatal and juvenile *pla2g6-inad* mice. We investigated survival, behavioural parameters, and histological analysis to assess therapeutic efficacy. The average lifespan of the model is reduced to approximately 14 weeks, with weight loss and behavioural decline from 9 weeks old.

Neuropathology studies showed neuronal loss and neuroinflammation in the brain and spinal cord, along with autophagic and lysosomal accumulation. A long term-study over 30 weeks demonstrated that neonatally administered AAV9.h*PLA2G6* gene therapy resulted in a significant improvement in all parameters measured including survival, weight, locomotor function, and neuronal counts in both the brain and spinal cord. Furthermore, the autophagic function was restored and lysosomal accumulation was significantly reduced. Adult administrations to symptomatic mice have thus far shown increased survival of 21 weeks on average and improved behavioural function. This study provides novel insights into INAD disease pathology and cellular dysfunction in the CNS and suggests an AAV9-based therapy has potential to enable effective treatment of INAD. Further clinical translation studies are being undertaken with our industrial partner, Bloomsbury Genetic Therapies Ltd.

OR82

Gene therapy for ALS and FTD: preclinical efficacy of AAV-hUPF1 optimized for clinical translation with improved vector genome and novel CNS capsid.

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ALS is a progressive neurodegenerative disease that affects neurons in the brain and spinal cord. Here, we present preclinical efficacy of AAV-hUPF1 in multiple models of ALS. hUPF1 is an RNA helicase that regulates NMD that we have optimized for AAV gene therapy of ALS.

At 4.9 kb, the optimized hUPF1 construct is 1.5 kb smaller than the original, thus offering AAV packaging and manufacturing benefits while demonstrating greater potency. When tested *in-vitro*, the optimized AAV-hUPF1 vector rescued toxicity in both the TDP-43 and C9orf72 iNeurons models, at lower MOIs than the original vector. Optimized AAV-hUPF1 also rescued pathophysiology of neurons derived from C9orf72 patient cell lines. Transduction in C9 patient-derived neurons indicated target engagement with down-regulation of C9 intron containing transcripts and known NMD targets. AAV-hUPF1 also showed *in-vivo* efficacy in a FUS knock in mouse model. Transduction of ~36% of spinal neurons was sufficient to drive significant rescue with surviving motor neuron counts comparable to WT level.

Furthermore, we developed a novel AAV capsid that expresses in neurons of both the brain and spinal cord when administered by ICM in mice. This ideal pattern was confirmed in NHPs with transduction that achieved levels sufficient to provide functional rescue in the *in-vivo* mouse model. The benefits of this efficient novel capsid for CNS delivery is aligned with our therapeutic goal of minimizing AAV exposure while maintaining strong transduction. Together, the evidence *in vitro* and *in vivo* with FUS, C9orf72 and TDP-43 models suggests that hUPF1 delivered by

MeiraGTx's proprietary vector is agnostic to genetic background and shows promise in treating both ALS and FTD, diseases with related pathophysiology.

OR83

Promoting functional improvement in ALS through myotropic AAV-mediated overexpression of α-Klotho

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Amyotrophic lateral sclerosis (ALS) leads to a gradual weakening and wasting of muscles due to the loss and deterioration of motoneurons. To preserve neuromuscular function in the $SOD1^{G93A}$ mouse model, we employed a synergistic approach to enhance α-Klotho secretion in skeletal muscles, targeting both motoneuron terminals and muscle endplates. α-Klotho is a pleiotropic protein that exhibits remarkable properties as a neuroprotective and myoregenerative agent.

In our previous studies, we intravenously administered AAV8 vectors at a dose of 3×10^{14} vg/kg to promote muscle secretion of α-Klotho in $SOD1^{G93A}$ mice. Overexpression of α-Klotho resulted in preserved motor function and strength, and delayed disease onset. The enhancement of neuromuscular function was evident through preserved amplitudes of the compound muscle action potentials (CMAP) and motor evoked potentials (MEPs). Furthermore, α-Klotho-treated $SOD1^{G93A}$ mice exhibited a higher number of surviving motoneurons and a significant reduction in glial reactivity within the spinal cord. These positive effects correlated with an increased occupancy of neuromuscular junctions and a preserved muscle mass.

Considering the substantial doses of AAV8 vectors required for therapeutic effectiveness, we transitioned to a myotropic AAV vector. By reducing the dose by 20-fold, we successfully achieved greater preservation of neuromuscular connectivity, improved motor performance, and enhanced strength in $SOD1^{G93A}$ mice. Notably, when administered to mice at a symptomatic stage, α-Klotho significantly decelerated the progressive decline of $SOD1^{G93A}$ mice. Finally, we have conducted comprehensive omics to uncover the precise mechanisms underlying the neuroprotective effects of α-Klotho in ALS and its role in preserving neuromuscular function.

Collectively, our findings offer compelling evidence that muscle-secreted α-Klotho holds the potential to enhance functional outcomes in ALS, paving the way for a novel therapeutic approach to address this debilitating disorder.

OR84

IND-enabling studies to support the clinical development of ATSN-201, a subretinally delivered, laterally spreading gene replacement therapy for X-Linked Retinoschisis (XLRS)

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Mutations in *RS1* are associated with X-linked retinoschisis (XLRS), one of the most common causes of juvenile macular degeneration in males. *RS1* encodes retinoschisin (RS1), a secreted protein that localizes primarily to photoreceptors and, to a lesser degree, bipolar cells that plays a role in the cellular organization of the retina. Affected males exhibit reduced visual acuity, schisis cavities within the macula, reductions in central retinal sensitivity and electroretinogram (ERG). To date, clinical trials delivering AAV vectors expressing RS1 (AAV-RS1) by intravitreal injection to XLRS patients have shown lack of efficacy and signs of inflammation. In addition, the presence of schisis cavities in the central retina has hampered the use of subretinal delivery using conventional AAV vectors due to safety concerns.

ATSN-201 (AAV.SPR-hGRK1-hRS1) is comprised of the novel AAV.SPR capsid containing the human retinoschisin (*hRS1*) gene. AAV.SPR transduces photoreceptors very efficiently and laterally spreads beyond the margins of the subretinal injection (SRI) bleb. This provides a tool to perform peripheral SRI without detaching the macula/fovea, thereby avoiding schisis cavities and to effectively deliver RS1 to both the peripheral and central retina. The purpose of this preclinical program was to generate safety and efficacy data to support use of ATSN-201 in a Phase I/II clinical trial. Two different animal models, RS1 knockout (RS1KO) mice and non-human primates (NHP), were used. In RS1KO mice, several studies were performed to fully characterize the animal model, identify the optimal vector construct, establish the minimum effective dose (MED) and therapeutic window, and assess safety in the disease background. Finally, the potential local and systemic toxicity of ATSN-201 was evaluated in a GLP toxicology study in NHP. In RS1KO mice treated between postnatal day 21-28, dose dependent improvements in retinal function, resolution of schisis cavities, and hRS1 expression in photoreceptor inner segments were observed. A hybrid pharmacology and toxicology study in RS1KO mice established the safety and efficacy of ATSN-201 in a disease setting and the GLP Toxicology study in NHPs confirmed that ATSN-201 was well tolerated at all dose levels, establishing the no observed adverse effect level (NOAEL) at the highest dose administered. Taken together, the preclinical studies summarized above demonstrated that subretinal administration of ATSN-201 was efficacious and well tolerated. A single, subretinal injection of ATSN-201 significantly improved retinal structure and function, supporting the clinical use of ATSN-201 for the treatment of XLRS. The ability of the novel AAV.SPR capsid to effectively deliver the RS1 to the central retina of primates following extrafoveal SRI represents a safer and potentially more effective treatment option for XLRS patients.

OR85

A single AAV packed with RNA base editor treats *OTOF*-mediated deafness

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Over 460 million people worldwide suffer from disabling hearing loss affecting 1-2% in 1,000 newborns with congenital hearing impairment. Mutations in the *OTOF* (otoferlin) gene encoding the multi-C2-domain protein otoferlin are the most common causes for auditory neuropathy induced hearing loss. Nonsense mutation of c.2485C>T (p. Q829X) in the *OTOF* gene located in exon 22 on the chromosomal region 2p22-23 is responsible for about 3% of all cases of recessive prelingual deafness in the Spanish population. Because the limited AAV cargo capacity would not be able to pack otoferlin gene (~6kb in size), previous studies adopted the dual-AAV (adeno-associated virus) gene therapy approach with each AAV vector containing a fragment of the large transgene to overexpress the otoferlin; however, the transduction efficacy of the inner hair cells (IHCs) was limited with only partial rescue of the auditory function. Here, we generated a a humanized OTOF-Q829X mouse model (*Otof* $^{Q829X/Q829X}$), consisting with part of the mouse *Otof* gene sequence replaced with human *OTOF* gene sequence. Next, we developed an enhanced mini-dCas13X RNA base editor (emxABE) and gRNA targeting *OTOF* (gOTOF) delivered by a single AAV variant with optimal transfection efficiency on hair cells (almost 100%) for the treatment of *OTOF*^{Q829X}, resulting in ~80% A-to-I (TAG>TGG and Q829>W) conversion efficiency in humanized *Otof* $^{Q829X/Q829X}$ mice. After a single Scala Media (SM) injection of emxABE targeting *OTOF*^{Q829X} (emxABE-T) to the postnatal day 0-3 (P0-3) of *Otof* $^{Q829X/Q829X}$ mice, nearly 100% of otoferlin expression was restored in the IHCs. The auditory function was rescued to near wild-type levels and the efficacy sustained for at least 7 months after a single injection. We also observed the restoration of auditory function in P5-7 and P30 *Otof* $^{Q829X/Q829X}$ mice via round window injection of emxABE-T. Furthermore, we demonstrated the increase of synapse of IHCs and the fully recovery of the latency and wave I amplitude in the emxABE-T treated mice for the first time when compared with other therapeutic strategies, suggesting that our better AAV transduction rate and higher editing efficiency strategy has the potential for the treatment of inherited hearing loss. Overall, our findings demonstrate not only a preferential therapeutic strategy for potentially curing OTOF-Q829X induced hearing loss, but also suggesting emxABE as the promising toolkits for the treatment of other monogenic diseases with premature termination codons. Clearly, our results suggest that clinical translation may potentially allow unaided hearing in people affected by *OTOF* mutations. To the best of our knowledge, we are the only group using base-editor strategy to potentially cure inherited deafness caused by *OTOF* mutations.

OR86

AAV1-hOTOF gene therapy trial for autosomal recessive deafness 9 (DFNB9)

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1.5 billion people worldwide live with hearing loss, and 430 million of them suffer from disabling hearing loss. Genetic defects cause hearing loss in one of 500 newborns. There is no pharmaceutical or biological treatment for hearing loss. Mutations in the gene *OTOF* cause autosomal recessive deafness 9 (DFNB9), manifested by the lack of sound detection or speech recognition from birth. Previous studies have demonstrated gene therapy to treat mouse models for DFNB9 with hearing recovery. No such study has been performed in human patients. We conducted a single-arm trial in children with DFNB9 by *OTOF* gene therapy.

We used a dual AAV approach in which the human *OTOF* cDNA was packaged into two adeno-associated virus (AAV) serotype 1 vectors for recombination to produce the full-length *OTOF* cDNA. A human hair cell promoter, Myo15, was used to drive the *OTOF* expression in affected hair cells. Five DFNB9 patients (2-6 years of age) with severe-to-complete hearing loss were enrolled in the trial. AAV1-hOTOF was unilaterally injected into the cochlea through the round window membrane (9×10^{11} and 1.5×10^{12} vg). The follow-up studies were performed on the patients for at least 13 weeks.

In the treated patients, no DLT (Dose-Limiting Toxicity) was observed. 39 adverse events were detected, with 95% (37/39) being grade 1 or 2, and 5% (2/39) being grade 3. Hearing was restored in four out of five injected patients. In the low-dose (9×10^{11} vg) group of one patient, hearing recovery was detected at 4 weeks post injection, with an average ABR (Auditory Brainstem Response) threshold of 64 dB, compared to over 95 dB before treatment. By 26 weeks, the average ABR threshold was further improved to 45 dB. In the best frequency (0.25 kHz and 2 kHz), the ABR thresholds were 35 dB. In the high-dose (1.5×10^{12} vg) group of four patients, hearing was recovered in three patients. At 4 weeks post injection, the mean average ABR threshold was 74 dB, compared to over 95 dB before treatment. By 13 weeks, the ABR thresholds were further improved to 55 dB. Speech perception testing was improved in 3 out of 4 patients with hearing recovery. Hearing was not recovered in one patient, who had a higher level of the neutralizing antibody before the treatment.

This is the first human study (ChiCTR2200063181) to show that gene therapy for DFNB9 is safe and without major adverse effect events. It recovered hearing with increasing efficacy over time in children who were otherwise unable to detect sound. The results support further development of gene therapy in DFNB9 patients, including expanded age groups and long-term observation.

OR87

Anti-CD45RC antibody leads to hepatocyte preservation after systemic AAV gene transfer in a humanized mouse model mimicking pre-existing anti-AAV T cell response

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Biotherapies using recombinant adeno-associated viral vectors (rAAV) showed a tremendous success for the treatment of genetic disorders during the last decade. However, rAAV immune toxicity issues related to the immune system activation that was not observed in animal models has recently been reported in patients injected systemically with high doses of viral vectors, leading in some cases to clinical trial holds. In these patients, the reactivation of preexisting anti-AAV T cells was shown to be associated to a hepatitis resulting from a cytotoxic elimination of transduced hepatocytes.

The absence of relevant preclinical models is a major limit for the prediction of success or failure of gene transfer protocols and for the development of less immunogenic rAAV vectors or relevant and specific immune suppressive drugs. We developed a humanized rodent model mimicking the cellular immune response described in patients. This model consists in engrafting NSG-HLA-A2+/HHD mice with human PBMCs collected from HLA-A2+ healthy donors with a preexisting anti-AAV8 cellular response. These humanized mice show an important weight loss when they are injected with both the rAAV8 vector and anti-AAV8 IFNγ-secreting human cells, as well as a better engraftment of human CD45+ cells (hCD45+) in the whole blood and the spleen. These mice also showed an inflammation in the liver with infiltrating mononuclear cells that are mainly proliferating CD8+ T cells in opposition to control groups.

Since this model appears relevant to test immunosuppressive strategies, we evaluated an immunomodulatory strategy using an anti-CD45RC antibody specifically targeting human naive and memory T cell subtypes. Control groups were injected with either the cells or the anti-CD45RC antibody only. Experimental groups were injected intravenously with the rAAV8 vector and the anti-AAV8 IFNγ-secreting human cells. In these groups, the anti-CD45RC antibody or its isotype were transiently administered for 3 weeks after cell delivery. Our results show a peripheral depletion of human CD45+ cells in the animals treated with the anti-CD45RC antibody resulting in less liver-infiltrating cells and less hepatic fibrosis compared to the isotype control group. Moreover, the vector biodistribution analysis in the liver of these mice show higher copy numbers after CD45RC treatment indicating the preservation of transduced hepatocytes.

Altogether, these results strongly suggest that our humanized mouse model is relevant to measure the impact of pre-existing anti-AAV T cells on gene transfer efficacy and that targeted T cell immunosuppression might prevent anti-AAV T cell reactivation after gene transfer.

OR88

Triple immunosuppression enhances vector transduction in periphery and CNS and promotes safety in Cynomolgus Macaques administered with high dose AAV vector

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Adeno-associated virus (AAV) based genetic medicines have been transformational in treating several genetic disorders. The widespread adoption of AAV vectors for in vivo gene delivery was based on the safety and efficacy of vector in targeting multiple tissues. However, clinical trials using AAV gene therapies have observed severe adverse events (SAE's) owing to the immune responses against the AAV capsid antigens and/or the transgene products. Furthermore, recent clinical trials have observed complement activation, thrombotic microangiopathy/Hemolytic Uremic Syndrome (HUS), and hepatotoxicity in subjects intravenously administered high AAV vector doses. The use of prophylactic or reactive glucocorticoids has been ineffective in completely ablating or reducing high-dose vector induced toxicity. Instead, interventions with additional immunosuppressants (IS) have provided clinical benefit however their effect on biodistribution and tissue immune cell infiltration is unknown. Here, we studied the effect of a triple IS (TIS) regimen combining methyl prednisolone (MP), rapamycin and rituximab on biodistribution in liver, kidney, heart, and CNS as well as the toxicity associated with the AAV9 gene transfer. Cynomolgus Macaques (N=3/group) received intravenously a high dose of AAV9 vector (5E13 vg/kg) expressing GFP from a ubiquitous promoter or vector along with the triple IS regimen. During the in-life phase of the study, clinical findings were observed in all animals that received vector without IS. Animals were lethargic and demonstrated signs of hindlimb dysfunction 30 days post vector infusion. In contrast, animals that received triple IS showed normal behavior with no functional deficits. Furthermore, the TIS regime enhanced vector transduction in liver, kidney, DRG, and spinal cord as compared to the animals in the no TIS group. The TIS regime also prevented hepatotoxicity with no significant transaminitis. In addition, animals had reduced DRG toxicity and cardiac troponin I levels were normal when compared to non-immune suppressed animals. Also, there were significant reductions in infiltrating mononuclear cells in tissues from animals that received the TIS regime. Finally, immunosuppressed animals had delayed blood vector clearance that appeared to correlate with higher peripheral and CNS transduction. Overall, the findings here support the use of these or similar regimens to reduce toxicity and increase safety of AAV gene therapy in the clinic. The added benefit of increased vector transduction upon TIS treatment may allow lowering of vector dose to improve the safety profile.

OR89

Immune tolerance promotion by LSEC-Specific lentiviral vector-mediated delivery of the transgene regulated by the stabilin-2 promoter

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Liver sinusoidal endothelial cells (LSECs) are specialized endocytic cells that clear the body from blood-borne pathogens and waste macromolecules through scavenger receptors (SRs). This function belongs to the ability of the stabilin-2 (STAB2) in mediating the binding of several ligands, including also coagulation products (as it mediates VWF-dependent FVIII endocytosis). Furthermore, current studies have demonstrated that FVIII is largely synthesized and secreted by endothelial cells (ECs), and specifically by liver sinusoidal endothelial cells (LSECs). However, LSEC role in immunity is only partially explored.

Since STAB2 is normally expressed by LSECs, we investigated whether the STAB2 promoter could be used to direct specific in vivo expression in LSECs upon LVs transgene delivery, thus resulting in a stable expression of the therapeutic protein due to the presence of natural regulating elements within cells and to the tolerance induction against the delivered transgene.

To investigate *in vivo* the role of LSECs in the modulation of the immune response against a transgene, we first studied STAB2 promoter (STAB2p) in driving transgene expression (GFP) after LV delivery into C57BL/6 or BALB/c mice, by immunofluorescence (IF) at several time points. Modulation of the immune response to the transgene regulated by the STAB2p was assessed by employing GFP-specific (Jedi) splenic CD8 T cells that were adoptively transferred in mice previously injected with LV carrying the GFP under the control of either STAB2p, the ubiquitous PGK or endothelial VEC promoter as a comparison. Expression of GFP was then verified after CD8 transfer by IF, while CD8 recruitment was assessed by flow cytometry. STAB2p activity has been further evaluated by injecting LV-carrying FVIII transgene in different strains of hemophilic mice.

After *in vivo* LV.STAB2-GFP delivery, GFP expression was mainly restricted to LSECs and stably maintained over time across mouse strains. When we performed the adaptive transfer with Jedi CD8 T cells, we observed GFP^+ cells in all livers of mice receiving LV.STAB2-GFP while no GFP^+ cells were detected in mice receiving LV.PGK-GFP or LV.VEC-GFP.

Moreover, this delivery system led to sustained transgene expression in hemophilic mice injected with a LV.STAB2-hFVIII which permanently restores FVIII activity in a mouse model of severe hemophilia A (HA), with no inhibitors formation.

In conclusion, the expression of GFP under the control of STAB2p resulted in a strong and specific expression into LSEC. Jedi GFP-specific CD8 employment confirmed the tolerogenic functions of LSEC able to prevent an immune reaction against the foreign protein. Additionally, these data reinforce our hypothesis that endothelial-specific expression driven by STAB2 promoter leads to stable FVIII expression and activity without the formation of neutralizing antibodies in immunoreactive HA mouse strains, confirming the power of this novel promoter. Overall, these data establish the suitability of STAB2p for long-term LSEC-restricted expression of therapeutic proteins for a potential application in gene therapy.

OR90

Immune modulation and the role of genetic diversity in translational animal models of AAV gene therapy

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The risk of immune-related adverse events (AE's) is a limitation to the use of adeno-associated virus (AAV) gene therapy. Capsid-specific antibodies induced after intravenous delivery have been implicated in complement activation leading to atypical hemolytic uremic syndrome and kidney injury, while capsid T cell responses are often associated with elevations in liver enzymes and a risk of liver injury. In addition, persistent high titer antibodies induced by AAV gene therapy prevent re-administration should transgene expression and efficacy wane. Inbred knockout murine models are widely used to demonstrate preclinical efficacy and biodistribution but fail to predict immune-related AE's. Thus, we believe there is a need for effective immune modulation regimens that blunt the adaptive immune response and improved animal models that recapitulate the human genetic diversity and immunological complexity.

Recently, immune modulation with Rapamycin and an anti-CD20 antibody that depletes B cells, has been used in several clinical studies to reduce the AAV adaptive response and potentially facilitate redosing. We utilized a mouse model of Pompe disease to test a redosing strategy. Acid α-glucosidase (GAA) knockout mice were given AAV8/Luciferase vector (1E11 vg /mouse) as the first dose and three months later an AAV8 vector expressing acid α-glucosidase (AAV8/GAA-3E11[1]vg/mouse). Immune modulation (rapamycin & murine anti-CD20) was administered for 30 days starting 1 week before each AAV dose. This modulatory regimen effectively depleted B cells from circulation and reduced antibody titers.

We believe one of the major limitations of this inbred mouse model is the lack of robust AAV specific T cell responses, highlighting the need for improved translational mouse models. To that end, we evaluated 16 genetically diverse collaborative cross (CC) mouse strains and eight founder strains each administered 3E11 vg/mouse of AAV8/GAA vectors and followed animals for 3-6 months post-AAV. GAA expression increased in the first weeks and plateaued after week four in the founder strains. In contrast, we saw >100-fold difference in collaborative cross strains with some strains having consistently high expression while others GAA expression significantly decreased overtime. GAA activity was associated with the vector genomes in liver, suggesting differences in biodistribution and/or liver transduction as one source of variation. In addition, we observed vast differences in the frequencies of splenic macrophage, Natural Killer cells, B cells along with naïve, memory and effector T cells between the genetically diverse CC lines. This immunologic diversity resulted in varying AAV- and GAA-specific antibody titers and the magnitude of the anti-GAA antibodies in serum was inversely associated with GAA expression. A robust GAA specific T cell response and low AAV-specific response was observed in distinct genetic strains, one of which had the lowest levels of GAA in serum.

This study demonstrates diverse mouse strains recapitulate many features of the response observed after AAV8 administration in humans. These strains may offer a translational model to test immune modulatory regimens in the future.

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Gene therapy for LGMDR9: preliminary results of a dose-escalation study

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Fukutin-related protein (FKRP) associated limb-girdle muscular dystrophy (LGMDR9) is a rare autosomal recessive disease with a prevalence of 1-4/200,000 in North Europa characterized by progressive proximal weakness of skeletal muscles and frequent cardiac and respiratory involvement. Onset of symptoms is generally during late adolescence in patients homozygous for the common L276I mutation. Loss of ambulation can occur on average 12 to 15 years later. Compound heterozygous patients may present a more severe phenotype with earlier onset of symptoms and loss of ambulation at an earlier age. Though muscle involvement at the hip and shoulder areas in LGMDR9 largely predominates, other muscles, such as respiratory muscles and the heart, can be affected by the dystrophic process. More than 50% of patients with LGMDR9 present with respiratory impairment while cardiac failure is reported in 20% of patients. Based on pre-clinical studies, a systemic gene therapy approach to this monogenic disease seems promising. Experiments in animal models showed full restoration at functional and histological level with doses greater or equal to 9.0E+12 vg/Kg. ATA-001-*FKRP* is a 2-stage, dose escalation and confirmatory study of ATA-100, an AAV serotype 9 carrying the human *FKRP* gene for the treatment of LGMDR9 (NCT05224505). The first two patients were treated in September 2022 and January 2023, respectively, and the study is anticipated to complete the recruitment of the 2 dose cohorts by February 2024. Following drug injection, patients reported transient nausea, vomiting, and grade 2 transaminase increase well controlled by immunosuppressant treatment. The primary objective of the dose escalation part of the study is to assess the tolerability of ATA-100 and to select the dose to be carried over to part 2 of the study, based on overall safety and biomarkers from the 3-month muscle biopsy. The primary objective of the randomized, placebo-controlled phase of the study (part 2) is to assess drug efficacy in improving or stabilizing muscular and respiratory functions at one year post treatment, compared to the placebo group and a natural history cohort. Safety, tolerability, muscle and cardiac MRI, and quality of life measures are secondary endpoints. Ten clinical sites in the US and Europe are anticipated to participate in this trial. The principal inclusion criteria are ambulant status, a forced vital capacity (FVC) of <80%, and a North Star Assessment for limb girdle-like muscular dystrophies (NSAD) <40. Patients with neutralizing anti-AAV9 antibodies are excluded. Status of the study, including preliminary clinical data and muscle biopsy results will be presented.

OR92

Expression of large dystrophins using AAVs and protein trans-splicing mediated by split inteins

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Gene replacement therapies mediated by Adeno-Associated Viral (AAV) vectors represent a promising approach for treating genetic diseases. Despite tremendous progress in capsid design and identification of novel serotypes with higher tropism and transduction activity, an important constraint on using AAVs is their modest packaging capacity (~4.7 kb). This size restriction can significantly limit their application for many genetic disorders involving large genes, such as Duchenne muscular dystrophy (DMD), which is caused by mutations in the 2.2 MB dystrophin gene that has an 11.2 kb coding region. Affected patients develop body-wide muscle wasting and die from cardio-respiratory failure in their late teens to early 30s. Our group has pioneered the development of miniaturized forms of dystrophin (including micro-dystrophins) that can fit within and be transported by a single AAV vector. Although these smaller dystrophins are surprisingly functional, many clones tested thus far are unstable or showed incomplete rescue of the dystrophic phenotype when tested in DMD animal models and in patients. These observations suggest that the expression of larger dystrophins with additional functional domains is necessary to fully protect from, or reverse, muscle pathophysiology.
Here, we present SIMPLI-GT (Split Intein-Mediated Protein Ligation for Gene Therapy), a novel method that allows the expression of large and stable proteins with high specificity and efficiency. This approach exploits the intrinsic ability of split inteins to ligate seamlessly multiple polypeptide fragments into a functional protein *via* a protein *trans*-splicing mechanism. We identified several split intein pairs that can efficiently join two or three fragments and generate, respectively, a large midi-dystrophin or the entire full-length dystrophin. In a proof-of-concept study, we show that the delivery of two or three AAV vectors results in a strong expression of large and functional dystrophins with a significant improvement in muscle histology and force development. Moreover, using the potent myotropic AAVMYO capsid, we demonstrate that a low dose of 2e13 vg/kg (10-fold lower compared to what is used in clinical studies) is sufficient to express large dystrophins in striated muscles bodywide with an almost complete physiological rescue. Our data show a clear superiority of large dystrophins over miniaturized forms when tested in young or very old dystrophic mice.

This novel strategy addresses emerging challenges of AAV-based gene replacement. If successful with DMD, it can be adapted to many other genetic disorders caused by loss-of-function mutations in large genes with a coding sequence beyond AAV packaging capacity.

OR93

XC001 gene therapy for the treatment of refractory angina: 6-month efficacy and safety from the phase 1/2 first-in-human study (EXACT Trial)

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Refractory angina (RA) is a debilitating chronic condition of advanced coronary artery disease in which patients have exhausted all available medical or surgical therapies and interventions. RA is characterized by high symptom burden and healthcare resource utilization, poor quality of life, and high rates of hospitalization. XC001 is a replication-deficient, recombinant adenovirus (Ad) 5 containing a human-derived cDNA/genomic hybrid and alternative splicing provides mRNA encoding for multiple isoforms of vascular endothelial growth factor (VEGF) which initiates angiogenesis to bypass coronary artery blockages. XC001 is administered under direct visualization by surgeons via a mini-thoracotomy to ischemic areas of the left ventricle identified by stress imaging studies and angiography which allows for the administration of lower vector genomes maximizing levels in the heart while minimizing systemic levels. Rodent biodistribution data demonstrated that direct epicardial administration resulted in localization of the vector to the left ventricular wall as compared to other highly perfused organs (liver, lung, spleen). XC001 was much more effective in the ischemic mouse hind-limb model at inducing angiogenesis and hind-limb blood flow than comparable vectors expressing only individual VEGF isoforms. XC001's increased ratio of expressed heparin-binding isoforms are designed to have a stronger local angiogenic effect due to their ability to bind to the extracellular matrix more tightly. Reported here are 6-month results from the phase 1/2 first-in-human, open-label, single-arm, sequential dose escalation study (EXACT) with the highest tolerated phase 1/2 dose of 10^{11} viral particles (n=29) demonstrating preliminary safety and signals of efficacy. The majority of subjects showed clinically meaningful improvements from baseline to 6 months post XC001 in the key endpoint of total exercise duration (TED) on a standardized exercise treadmill test read by a blinded core lab (mean (SD) increase of 1.4 (2.3) minutes, p=0.003). Mean (SD) change in angina episodes at 6 months showed a reduction of 6.7 (8.1) episodes (p<0.001) over a 2-week period prior to each visit while at the same time nitroglycerin use decreased (-3.8 (6.9), p=0.008). Canadian Cardiovascular Society (CCS) Class, a measure of angina symptoms, improved in 81% of subjects. There was a 14% reduction (p=0.02) in total myocardial perfusion deficit on Positron Emission Tomography (PET) observed at 6 months. An assessment of treatment success across 5 key endpoints (TED increase of >1 minute, angina episode reduction of >30%, nitroglycerin reduction of >30%, >1 class improvement in CCS, and >10% reduction in TPD) showed 93%, 67% and 40% of subjects meeting ≥2, ≥3 and ≥4 endpoint criteria, respectively. No serious adverse events (SAEs) were considered related to XC001 and SAEs related to the administration procedure (38%) were expected and occurred within 2 weeks of dosing. In summary, multiple EXACT study efficacy endpoints demonstrated that intramyocardial administration of XC001 was associated with improvements in angina symptoms and exercise capacity in RA subjects while cardiac imaging measured by PET provided supportive evidence of a biologic effect.

OR94

Evaluating the therapeutic potential of CRISPR/Cas9 based gene editing in a newly developed inducible multi-systemic myotonic dystrophy mouse model

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Myotonic dystrophy type 1 (DM1) is a multi-systemic genetic disorder caused by a mutation in the dystrophia myotonica protein kinase (DMPK) gene forming pathogenic expanded CUG repeats in multiple tissues thereby compromising multiple organs. Due to the complexity of the disease, its pathogenic mechanism is not yet well elucidated. The lack of adequate animal models mimicking the multi-systemic disease phenotype is a current challenge. We therefore developed a multi-systemic DM1 mouse model allowing regulated expression of the pathogenic CUG_{exp} transcripts in multiple tissues, mimicking the clinical manifestations of DM1. Transcriptomic analysis on this mouse model helped identify novel genes commonly impacted in all three tissues (muscle, heart, brain), hinting at their potential use as biomarkers to aid in therapy. Though various therapeutic approaches for DM1 are being explored, currently no effective cure or treatment is available. Recent advances in gene-editing therapies based on CRISPR/Cas9 hold great promise for the treatment of genetic disorders such as DM1 with unprecedented precision. Hence, in this current proof of concept study, we explored the use of CRISPR/Cas9 mediated gene editing using a dual gRNA-based strategy capable of excising the CTG_{exp} repeats in the human DMPK locus. To identify and validate the best dual gRNA combination, we designed and developed 9 different gRNA combinations to excise the expanded repeat region efficiently. Co-expression of selected top 3 dual gRNAs with Cas9 *in vitro* on human DM1 iPSC-derived myogenic cells, displayed efficient correction. We also used CRISPR/Cas9 to successfully correct the disease phenotype in human DM1 iPSC-derived cardiomyogenic cells consistent with robust excision of the pathogenic repeat sequence. Subsequently, we administered the CRISPR/Cas9 components using AAV9s vector to our newly developed mice model. Our results show successful excision of the repeat region with reversal of DM1-specific alternative splicing markers and decrease in ribonuclear foci formation. Taken together, the current study demonstrates a newly developed multi-systemic DM1 mouse model closely mimicking the disease phenotype that serves as a suitable model to study both mechanistic and therapeutic interventions. Most importantly, CRISPR/Cas9 was successfully used after systemic *in vivo* delivery to achieve partial correction of some of the quintessential DM1 disease markers, underscoring its potential for DM1 treatment.

Poster Presentations

P001

AAV-STARR-Seq reveals novel enhancer/promoter combinations in a complex *in vitro* model of the human retina

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AAV-STARR-Seq (Self-transcribing active regulatory region sequencing in the AAV context) is a powerful tool to identify novel regulatory elements (promoter/enhancer combinations) for transcriptional control in therapeutic AAV vectors. We previously employed AAV-STARR-Seq to screen for regulatory elements that drive expression in the mouse brain. Here, we tested the capability of AAV-STARR-Seq to identify enhancer candidates which are active in the recombinant AAV genome context after transduction of human iPSC-derived retinal organoids, a complex *in vitro* system comprised of several distinct cell types. To enable the screening, we demonstrated I) the ability of retina-tropic AAV capsids to target different cells in the organoid and II) that cell-type specific expression can be achieved employing previously published promoters in the AAV expression cassette. In a next step, we *in silico*-designed a library of 10800 enhancer and promoter candidates by integrating open chromatin and enhancer-associated histone modification datasets. This library was cloned into a STARR-Seq expression cassette and packed into AAVs which were subsequently applied to matured retinal organoids. In the NGS-based analysis, we identified several elements with potential enhancer function as defined by an increased RNA/DNA ratio. Importantly, for the AAV-STARR-Seq technology, we observed a critical relationship between library complexity and target cell number as a precondition for a successful screening campaign. In summary, we show for the first time that AAV-STARR-Seq can be used in a complex human *in vitro* model system of the retina. Our findings hold promise for the identification of novel regulatory elements for gene therapeutic approaches in healthy but also diseased organoids carrying patient-specific mutations.

P002

Gene therapy of bladder dysfunctions by a replication-incompetent recombinant HSV-1 vector expressing the light chain of botulinum neurotoxin F in C-type afferent bladder neurons.

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EG110A is a non-replicative recombinant herpes simplex virus type 1 (HSV-1)-derived vector that expresses the light chain of the botulinum neurotoxin F (BoNT/F-LC) driven by the human calcitonin gene-related protein (hCGRP) promoter, designed to achieve long-term sensory neuron-selective transgenic expression in bladder afferents. The mechanism of action of EG110A is based on the natural ability of HSV-1 to infect peripheral sensory neurons, and to express the BoNT/F-LC inside the targeted neurons, leading to the cleavage of the VAMP2 protein that is essential for neurotransmission. The hCGRP promoter intends to reinforce the selectivity of the vectors for C-fibre bladder afferents, whereas the introduction of the transgene in the LAT region of the vector genome promotes long-term expression. The aim for EG110A is to provide a long-term efficacy, possibly lasting several years, for the treatment of neurogenic detrusor overactivity (NDO) and other lower urinary tract dysfunctions such as OAB (overactive bladder) or interstitial cystitis/bladder pain syndrome (IC/BPS), while preserving the ability of the bladder to contract and not cause urinary retention.

Here we describe the construction and properties of EG110A and demonstrate both the safety and the ability of this vector to inhibit bladder sensory C fibres in an acute intravesical capsaicin rat model. Capsaicin is an irritating molecule acting on sensory nerves via TRPV1 vanilloid receptors mainly present in type-C afferent neurons. Acute intravesical capsaicin is a suitable model to assess afferent hyperactivity mimicking the pathophysiology of NDO and OAB. The main finding of this study is that EG110A counteracted bladder contractions elicited by capsaicin-mediated activation of TRPV1 receptors in C-fibre bladder afferents, as seen by a significant decrease of the frequency of micturition and increase of the bladder capacity. Due to its selective effect on C-fibres EG110A inhibits the reduction of inter-contraction intervals (ICI) induced by capsaicin, without any change in normal bladder function. In addition, EG110A does not lead to urinary retention. Five weeks after vector injection in the bladder wall, rats were sacrificed, and transgene expression was assessed by ddPCR and RNAscope in L6/S1 DRG.

Concluding message

HSV1-based non replicative vectors are being developed as an efficient tool to achieve long term expression of a transgene in sensory neurons. EG110A is a promising approach to treat C-fibre related NDO and other lower urinary tract disorders. EG110A has the potential to provide long term efficacy without adverse effects on voiding. Finally, these results show for the first time that intraneuronal expression of BoNT/F-LC can have a significant therapeutic potential. Noteworthy, beyond bladder dysfunction, the mode of action of EG110A makes this vector potentially suitable to treat any pathology driven by type C sensory neurons.

P003

Anellovectors: a novel functional gene delivery platform based on commensal human anelloviruses demonstrates transduction in multiple cell and tissue types

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Anelloviridae is a highly diverse family of non-enveloped negative-sense ssDNA viruses that infect vertebrates. While anelloviruses are a ubiquitous component of the human virome, they evade induction of humoral immune response and appear to be apathogenic in humans. These properties make anelloviruses an attractive candidate for harnessing the human virome for the next generation of genetic medicines. Here we report the development of a completely novel gene therapy class termed Anellovectors. Vectorization of anelloviruses is enabled through development of the Self-Amplifying Trans-complementation of a Universal Recombinant Anellovector (SATURN) production system, which relies on a self-replicating plasmid to provide viral proteins in trans that drive replication and packaging of vector genomes. Using the SATURN system, capsid protein-dependent particles that encapsidate ssDNA vector genomes are produced. Like most mature viral vector systems, the vector genome is devoid of viral genes and only carries therapeutic or reporter transgenes. We validated these particles by isopycnic centrifugation, DNase-protected qPCR, next generation sequencing, and electron microscopy. Furthermore, we demonstrate packaging of a vector genome from a single anellovirus with capsids from multiple anellovirus species, suggesting we can build a universal vector platform that takes advantage of the remarkable diversity of anelloviruses. In vitro transduction was validated by detection of an eGFP reporter and detection of vector genomes in nuclei by in situ hybridization. The expression of an eGFP reporter was validated in mouse studies in a variety of tissues using different routes of administration. To our knowledge, this is the first report of a functional anellovirus-based gene therapy vector. Anellovectors have the potential to deliver safe, redosable, and potent therapeutics, helping to expand the reach of programmable medicines.

P004

AnelloBricks: a novel, cell-free, in vitro assembled viral vector system based on commensal human anelloviruses offers a highly modular, versatile, and scalable manufacturing platform solution to genetic medicines

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Viral vectors show great promise for efficient gene transfer and therapeutic drug delivery. However, safety concerns arising from immunogenic and cytotoxic effects as well as difficulties in manufacturing scalability and productivity remain key challenges for their use. Here we report the development of ana cell-free, in vitro assembled genetic medicines platform offering a safer and modular approach to treating a broad range of diseases across diverse therapeutic modalities based on human-derived anelloviruses. Anelloviruses are ssDNA viruses that are ubiquitously commensal in human populations and are not known to be the etiological agents of any disease in humans. Anelloviruses are extremely genetically diverse and appear to evade the immune system. The non-pathogenic and commensal nature of anelloviruses make them a prime candidate for use in next generation genetic medicines. This novel in vitro assembled genetic medicines platform is comprised principally of two components: recombinant protein and nucleic acid payload (e.g. DNA, RNA), offering payload versatility while also reducing the complexity of manufacture to conventional and readily scalable production systems. This modular, versatile, and scalable approach has the potential to address the limitations of current cell-based viral production systems and improve access to patients in need by dramatically reducing the cost to manufacture.

P005

Gene therapy for Myasthenia Gravis

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Amplo Biotechnology is a gene therapy company focused on enhancing the neuromuscular junction (NMJ). While studying mechanisms to enhance neuromuscular junction function, we have uncovered a therapeutic strategy to manage myasthenia gravis using gene therapy. Our work describes using an adeno-associated virus designed for improved safety and efficacy to protect and enhance the NMJ in pre-clinical myasthenia gravis study.

P007

REKLAIM - A novel phase 1b gene therapy clinical trial using FBX-101 (AAVrh10-hGALC) administered intravenously to patients who received haematopoietic stem cell transplantation for the treatment of Krabbe Disease

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Krabbe disease is a severe neurodegenerative disorder caused by galactocerebrosidase (GALC) deficiency. If left untreated, the infantile (IKD) and late infantile form (LIKD) lead to death by an average of 2 and 6 years of age respectively. Asymptomatic patients are diagnosed through family history or newborn screening, which is available in 11 US states. Haematopoietic stem cell transplantation (HSCT), the current standard of care, improves outcomes in asymptomatic patients, but the benefit is limited by progressive peripheral neuropathy resulting in motor weakness and decline. FBX-101 is an intravenously administered AAVrh10-hGALC vector designed to target higher GALC levels in the peripheral nerves.
REKLAIM is a Phase 1b dose-escalating clinical trial to evaluate safety and efficacy of FBX-101 administered systemically in subjects with IKD and LIKD previously treated by HSCT. Subjects in Cohort 1 receive a single IV infusion of FBX-101 at a low dose of 1.6×10^{13} vg/kg >90 days post-HSCT. The immune suppression regimen (ISR) is individually adjusted according to the immune status of the patient at the time of administration after review by the Immunosuppression Regimen Committee (ISRC). There is a trial evaluation period of 2 years post-administration, with an additional 3 years of follow-up. We report on the safety and preliminary efficacy of the first 2 subjects treated in REKLAIM.

The first subject was diagnosed with IKD and transplanted as a neonate. At screening he was 4 years old and his chimerism was 32%, resulting in borderline GALC levels. His ISR consisted of Rituximab (one dose), Sirolimus and Prednisolone. After FBX-101 administration, GALC activity in plasma increased to supraphysiologic levels until Day 120 when GALC was noted to drop but remained 7-fold above baseline and within the normal range. Psychosine levels decreased after dosing. The subject developed antibodies to AAVrh.10 but there were no liver enzyme elevations and no treatment-related serious adverse events with complete tapering of ISR. Motor outcomes (PDMS-2) have significantly improved after 6 months of follow-up.

The second subject was diagnosed through newborn screening and received HSCT on Day 32 of life; the patient fully engrafted and immune reconstituted by the time of REKLAIM screening at 18 months of age. His ISR also consisted of Rituximab (two doses), Sirolimus and Prednisolone. The subject maintained normal liver function tests and no treatment-related serious adverse events have developed after 30 days of follow-up.

P008

Results of a phase 1 open-label, dose escalation study of gene therapy with AAV2-hAQP1 as treatment for radiation-induced xerostomia and parotid gland hypofunction

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In radiation-induced xerostomia, the normal architecture and function of salivary glands are significantly disrupted or destroyed. AAV2-hAQP1 vector expresses the human Aquaporin 1 (hAQP1) gene delivered using the AAV2 capsid. When hAQP1 is expressed in cells of the disrupted glands, the cells become permeable to water. Water flows down the hydrostatic pressure gradient through the salivary duct and into the mouth.

In this study, AAV2-hAQP1, at doses ranging from 1 × 1011 vg/gland to 3 × 1012 vg/gland, was administered to one (unilateral) or both (bilateral) parotid glands via intraoral, retroductal cannulation of Stensen's duct. Key inclusion criteria for study participants were a history of head and neck cancer, a minimum of five years since final radiotherapy treatment (two+ years if HPV+), the presence of grade 2/3 late xerostomia, no evidence of cancer recurrence or second primary, and abnormal parotid gland function. Key exclusion criteria were a history of autoimmune disease affecting the salivary glands and a hemoglobin A1c greater than 7%.

Safety parameters included assessments of adverse events, physical examination observations, clinical laboratory results, and electrocardiogram findings. Efficacy assessments included participant completion of the Xerostomia-specific Questionnaire (XQ) and Global Rate of Change Questionnaire (GRCQ). The XQ is a patient-reported outcome measure consisting of eight symptom-specific questions that the participant rates from 0 (not present) to 10 (worst possible). The sum of all ratings (0-80) provides an overall measure of symptom burden. The GRCQ is a patient-reported outcome tool that has been adapted for xerostomia. The GRCQ first asks the participant if their dry mouth is "better", "worse", or "about the same" following treatment. If the participant reports "better" or "worse," they are then asked to rate the degree of change on a scale from 1 to 7, with 1 being the smallest change and 7 being the greatest change. A score of 2 or above is important to the patient. To evaluate the biologic activity of AAV2-hAQP1, change from baseline to Month 12 in whole saliva flow rate was assessed.

A total of 24 adults were enrolled in the study, with twelve participants treated unilaterally and twelve treated bilaterally. AAV2-hAQP1 was safe and well-tolerated at all dose levels, with no treatment-related serious adverse events or dose limiting toxicity.

At Month 12, 16 of the 24 participants had an improvement of ≥8 points in XQ score and 18 of the 24 reported important improvement in xerostomia symptoms relative to baseline on the GRCQ. In bilaterally treated participants, the average percent increase in unstimulated whole saliva flow rate at Month 12 post-treatment relative to baseline was 82.1%. On all efficacy measures, greater improvement was observed in bilaterally treated participants than in those treated unilaterally.

Treatment with AAV2-hAQP1 was safe, resulted in important improvements in symptoms of radiation-induced xerostomia, and increased unstimulated whole saliva flow rate. Based on these promising results, a Phase 2 study has been initiated.

P009

Two year muscle MRI observations from a phase 1b trial of fordadistrogene movaparvovec (PF-06939926) for Duchenne muscular dystrophy (DMD)

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Fordadistrogene movaparvovec (FM, PF-06939926) is an adeno-associated virus serotype-9 (AAV9) gene-replacement construct containing a mini-dystrophin being developed for DMD, aiming to restore functional protein to muscle. Magnetic resonance imaging (MRI) is a non-invasive tool to monitor disease progression. We present 2-yr quantitative MRI measurements from 16 ambulatory DMD participants from a phase 1b, multicenter, single-arm, open-label trial (NCT03362502). FM was administered as one iv dose (2E14 vg/kg). An external reference (ER) cohort was derived from DMD participants (n=48) with ≥1 yr of randomized domagrozumab treatment (active→placebo, placebo→active, or active→active) from trial NCT02310763 and met eligibility criteria for this trial. Whole-thigh MRI scans were acquired at baseline, 6 months, 1-yr, and 2-yrs to evaluate changes in muscle volume (MV) and fat fraction (FF). Mean %change from baseline (CFB) in MV (mean±SD) was 9.0±13.5% (1-yr) and 7.4±16.1% (2-yrs) (FM) vs 2.7±8.3% after 1-yr and 2.4±12.1% after 2-yrs (ER). Mean CFB in FF was 0.6±2.8% at 1-yr and 3.3±3.2% at 2-yrs (FM) vs1.6±2.6% after 1-yr and 3.3±4.1% after 2-yrs (ER). Younger participants (6-7 yrs old) had larger changes in %CFB in MV: FM (n=6): 20.7±13.4% (1-yr) and 22.8±10.9% (2-yrs) vs ER (n=23): 4.5±8.4% (1-yr) and 6.0±13.6% (2-yrs). Two-years after dosing with FM, participants with increases in thigh MV had higher functional performance, including North Star Ambulatory Assessment and 10-m run time. Thigh MRI findings are further supported by evaluating upper limb MRI measure. After dosing with FM, quantitative muscle MRI measurements show favorable changes up to 2-yrs that correlate with functional outcomes.

P010

RESKUE, a first in human phase I/II clinical trial of FBX-101 (AAVrh10.GALC) intravenously administered after UCBT for the treatment of Patients with infantile Krabbe disease

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FBX-101 RESKUE is a novel and first in human AAV intravenous therapy that is administered after myeloablation and immune suppression for umbilical cord blood transplantation (UCBT) to treat infantile Krabbe disease (IKD) avoiding antibody response. 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 in average by 2 years of age. Currently, asymptomatic neonates diagnosed because of family history or newborn screening, are treated with UCBT within the first month of life. The UCBT graft addresses mostly the demyelination of the brain, but the motor function continues to decline due to progressive peripheral neuropathy with death by teen years. RESKUE is a Phase 1/2 dose-escalating intravenous gene therapy to evaluate safety and efficacy of FBX-101 administered IV 21-60 days after UCBT. The myeloablation and immune suppression overrides the antibody response to the vector's capsid and the healthy donor UCBT has normal GALC preventing antibodies to the transgene. Additionally, UCBT is immunologically naïve to AAV and has an immature immune system. We report results on the first two subjects treated in cohort one (low dose) that received an IV infusion of FBX-101 of 1.6 $x10^{13}$gc/kg (low dose), 25 and 29 days after UCBT respectively. FBX-101 was well tolerated, with no treatment-related serious adverse events up to 12 and 18 months after administration. No antibodies to the capsid or transgene developed, and both subjects engrafted with full chimerism. We report supraphysiological increases in plasma GALC and CSF GALC as well as psychosine drop below the levels of detection. Additionally, when compared to healthy and transplanted subjects, we demonstrate normalization of gross motor skills using the PDMS II with both subjects walking independently. White matter growth in the brain was quantitated using brain MRI diffusion tensor imaging tractography, demonstrating better trajectory than most asymptomatic transplants and overlapping with healthy controls. In summary, FBX-101 after UCBT leverages themyeloablation and immune-suppression after UCBT, resulting in efficient AAV transduction and providing supraphysiological GALC enzyme to support brain myelination and gross motor development.

P011

Preclinical development of SENS-501 as a treatment for the autosomal recessive non-syndromic deafness 9 (DFNB9) using an adeno associated vector-based gene therapy

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Congenital sensorineural defects are one of the most severe forms of congenital impairment, heavily impacting the life of the patients and their ability to communicate with others. Among this vast family of communicating disorders, the non-syndromic autosomal recessive deafness 9 (DFNB9) is one of the most common forms of congenital deafness, accounting for up to 8% of cases. This severe-to-profound auditory neuropathy is caused by a biallelic loss of function in the *OTOFERLIN* gene (*OTOF*), which encodes for a calcium sensor protein involved in the neurotransmitter release at the presynaptic level between the inner sensory hair cells (IHC) and the spiral ganglion neurons. So far, the only medical solution is the cochlear implantation, which improves hearing to some degree, but still has a lot of limitations. To address this unmet medical need, we developed SENS-501, a dual AAV (adeno associated virus) hybrid approach using two different recombinant vectors each containing one half of the *OTOF* cDNA. This strategy was tested on congenitally deaf DFNB9 mutant mice by injecting SENS-501 in the inner ear through the round window membrane using different doses. The reversal of the deafness phenotype in our knock-out mouse model was evaluated through multiple auditory and behavioural tests. Auditory brainstem response (ABR) recordings showed significant lowering of the thresholds along the auditive spectrum after intra-cochlear injection, showing a durable improvement of hearing in a dose-responsive manner analyzed as early as three weeks post-injection and that lasted at least ten months. These mice were also submitted to a startle-test protocol experiment to confirm their ability to efficiently process sound similarly to wild-type mice. We were able to detect a restoration of their startle reflex ability when exposed to randomized sudden loud noises. We also evaluated the vestibular function of treated mice by analyzing their locomotion patterns in an open-field arena. Data indicated that our approach did not show any significant increase in circling behaviour when compared to control mice. We performed a similar set of experiments in non-human primates (NHP) after SENS-501 intracochlear injections using a surgical method and our proprietary medical delivery device envisioned in clinical trial. Surgery and SENS-501 did not affect ABR thresholds, similar to what was observed in mice. Immunohistochemistry experiments were performed in both mice and NHP, demonstrating effective recombination and selective expression in IHCs of the full-length therapeutic protein and its flag-tagged counterpart. A 3-month GLP toxicology and biodistribution study following a single intra-cochlear injection was conducted in NHP with two doses of SENS-501. Biodistribution data indicated that the vast majority of the vectors remained in ear structure that received the injection. SENS-501 shedding in fluids showed a limited dissemination of the vectors and all the shedding samples decreased over time. The two doses of SENS-501 were well tolerated and did not induce any macroscopic/organ weight changes or local/systemic microscopic findings, Therefore, our nonclinical pharmacology, biodistribution, and safety studies support the clinical development of SENS-501 for hearing loss due to genetic OTOFERLIN protein deficiency.

P012

Dyno bCap1: single-cell characterization of CNS transduction by intravenously administered AAV capsids in non-human primates

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1: Dyno Therapeutics

Highly efficient AAV capsids that broadly transduce the CNS will unlock gene therapies for currently untreatable neurological diseases. Dyno bCap 1 is an intravenously administered AAV capsid with field-leading potential for gene delivery to the brain. Relative to AAV9, Dyno bCap 1 exhibits 10-fold liver detargeting and 100-fold CNS transduction in Cynomolgus monkey, 80-fold enhanced CNS transduction in African Green monkey, and comparable production efficiency.

In this study we characterized Dyno bCap 1 gene delivery to the Cynomolgus monkey brain using single-nuclei RNA sequencing (snRNA-seq), and compared its performance to that of novel AI-designed capsids and an external engineered capsid. snRNA-seq of >200,000 nuclei revealed substantial transduction by Dyno bCap 1 and novel AI-designed capsids across the brain, including in neurons, astrocytes, and oligodendrocytes. In contrast, AAV9 transduced very few cells. As measured by overall transduction averaged across two animals, Dyno bCap 1 outperformed the external engineered capsid, whereas AI-designed capsids demonstrated substantial improvements in CNS transduction even over Dyno bCap 1.

In a two capsid validation study comparing Dyno bCap 1 and AAV9, each injected intravenously at 1e13 vg/kg, Dyno bCap 1 demonstrated transduction of up to 20% of neurons and up to 15% of all cells throughout the brain when measured by RNAscope. Across brain regions, the percentage of cells transduced as quantified by automated histology was highly correlated to the number of transduction events resolved by barcoded NGS. The brain regions with the highest rates of transduction included the substantia nigra, motor cortex, cerebellum, and ventral horn of the spinal cord, with potential relevance for indications such as amyotrophic lateral sclerosis (ALS), Parkinson's disease, Friedreich's ataxia, and spinal muscular atrophy (SMA). In contrast, AAV9 co-injected at the same dose transduced very few cells throughout analyzed brain regions. In dorsal root ganglia, Dyno bCap 1 and AAV9 transduced cells with similar efficiencies.

Our results showcase the power of AI for designing AAV capsids that are dramatically more efficient and precisely targeted for in vivo delivery, unlocking new possibilities for treatment of neurological diseases with gene therapy.

P013

Long-term follow-up of a liver-directed gene therapy clinical trial for mucopolysaccharidosis type VI

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Mucopolysaccharidosis type VI (MPS VI) is an autosomal recessive lysosomal storage disease due to arylsulfatase B (ARSB) deficiency resulting in multisystem accumulation of glycosaminoglycans (GAG). Current treatment for MPS VI is based on weekly infusions of enzyme replacement therapy (ERT). However, ERT requires life-long parenteral infusions at high costs and considerable inconvenience for patients and families. This formed the basis for the development of liver-directed gene therapy based on a single intravenous administration of a recombinant adeno-associated virus (AAV) vector. Herein, we report the long-term follow-up on MPSVI patients enrolled in the high dose cohort of a phase 1/2 open label, dose-escalation, multicenter clinical trial (NCT03173521) investigating the safety and efficacy of intravenous infusions of a recombinant AAV serotype 8 vector expressing the human arylsulfatase B (*hARSB*) gene under the control of a liver-specific promoter (AAV2/8.TBG.hARSB). While patients at the low and intermediate dose cohorts had to restart ERT because the vector-reduced expression of ARSB was not sufficient to maintain urinary GAG unaffected after ERT discontinuation, patients in the high-dose cohort (6×10^{12} gene copies/kg, n=4, mean age at administration: 8.75 ± 2.50 years) showed sustained serum ARSB and a modest urinary GAG increase that did not reach a concentration at which ERT reintroduction was needed. In this high-dose group, all four patients did not show clinical deterioration for up to 3 years after gene therapy. However, in one out of four patients, ERT was restarted because of elevation of GAG about 2.5 years after gene therapy. The three patients who remained without ERT showed no relevant changes in endurance, as evaluated by the 6-minute-walk and the 3-minute stair-climb tests. Although one participant showed an asymptomatic mild reduction, pulmonary function as measured by forced vital capacity and forced expiratory volume in 1 second, did not vary after gene therapy, despite ERT interruption. Although liver and spleen size remained within normal reference ranges, one participant showed increased in the centile of the liver size and two participants showed increased centile of the spleen size compared to baseline. In all three participants who remained without ERT, left ventricle ejection fraction, interventricular septum thickness, aortic, mitral, and tricuspid valve leaflet thickness did not change significantly. No late emergent safety events were recorded in any of participants. Patients at the high dose showed sustained ARSB expression in the 35-56% range of mean normal values for at least 3 years post-gene therapy. Interestingly, one of the patients was five- and two patients were ten-year old at the time of treatment, suggesting that liver-directed gene therapy can be performed in patients of five years or older without loss of transgene expression due to hepatocyte proliferation. Although longer follow-up in more patients is warranted, these results confirm that a single intravenous administration of AAV2/8.TBG.hARSB is safe and results in sustained expression of serum ARSB together with only a mild increase in urinary GAG in the high dose-group, thus supporting liver-directed gene transfer as a treatment for MPSVI.

P014

Combining multiple capsid engineering approaches to develop AAV vectors with novel properties

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Adeno associated virus (AAV) tissue tropism is a property determined by the protein capsid. Naturally occurring capsids have diverse sequences, especially in surface exposed variable regions, and display a range of tropisms for different cell types. However, none are specific for any cell or tissue type. As AAVs are widely used as gene therapy vectors it would be desirable to engineer specificity into the capsids so that diseased cells could be targeted and other tissues avoided. A number of methods have been described to enhance tropism (as well as overall transduction) of vectors. For regions of the capsid surface where protein interactions are understood, rational engineering in the form of amino acid substitution can be undertaken to modify vector properties or larger sections of sequence swapped between capsids. More commonly, directed evolution is used as an unbiased approach to enhance capsids with new properties. Additionally, proteins (or other ligands) of known function can be fused or coupled to the capsid surface to add novel properties to the vector. Here we present a multi-tiered approach where we combine rational mutagenesis, directed evolution, and the fusion of protein binding domains to the capsid surface to discover new gene therapy vectors.

Starting with an AAV9 vector, we mutated specific residues in the three-fold spike region on the surface to introduce liver detargeting. In mouse studies these mutations resulted in significant liver detargeting while not affecting tropism to other tissues. However, in NHP these same vectors showed reduced transduction in multiple tissues in addition to the liver. To overcome this, we then built multiple peptide insertion libraries into different surface exposed loops in the liver detargeted vector and selected for capsids which continued to show low transduction of liver relative to AAV9 but which recovered the ability to transduce other organs of interest in NHPs. Three rounds of selection were performed in NHPs with barcoded capsid libraries in order to select winners. This was followed by in *vitro*, *in vivo*, and structural analysis with the winning capsids. Using this approach, we were able to identify multiple novel capsids fitting our selection criteria. In parallel we developed an optimized AAV9 scaffold to fuse protein binding domains to the surface of capsids to allow for enhanced tropism on a cell specific level. We investigated multiple fusion insertion points, linkers, and approaches for stabilizing surface peptide loops. Using Designed Ankyrin Repeat Proteins (DARPin) selected to bind transferrin receptor as a model for protein insertions on the capsid surface we were able to redirect the vectors *in vivo*. Finally, we merged these approaches to investigate if combining rational mutagenesis, library-selected peptide insertions, and fused protein binding domains was feasible within a single vector.

P015

A novel AAV capsid demonstrating high specificity to motor neurons with enhanced transduction in macaque and marmoset monkeys and mice following systemic administration

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Motor neuron diseases (MNDs) are debilitating conditions characterized by progressive degeneration of upper and/or lower motor neurons. Patients affected by MNDs suffer from unrelenting decline of muscle strength, resulting in paralysis, respiratory failure, and even fatality. In this regard, recent significant advancements in adeno-associated virus (AAV) vector technologies have opened new avenues for the treatment of a wide range of CNS disorders, including MNDs. The clinical use of the FDA-approved wild-type AAV9 product for the treatment of an MND has demonstrated promising outcomes, highlighting the potential of AAV gene therapy as a therapeutic option for MNDs. While systemic administration of AAV vectors holds promise as an optimal route of administration, the use of the wild-type AAV9 and other commonly used serotypes poses challenges, including the need of high vector doses (>10^{14} vg/kg) potentially causing serious adverse effects, limited efficacy in crossing the blood-brain barrier, inadequate target cell specificity, and the absence of conserved tropism across different animal species that facilitates clinical translation. Therefore, there is an unmet need of an engineered AAV capsid with superior motor neuron transduction with high cell type specificity across different species. Here we show that AAV-CGN2, a novel AAV capsid identified using the TRAnscription-dependent Directed Evolution (TRADE) platform, exhibits significantly enhanced transduction in both upper and lower motor neurons with high cell type specificity following systemic vector administration. Importantly, these exceptional properties are preserved across multiple species, including cynomolgus macaques, rhesus macaques, marmosets and mice. In this study, animals of all the four species received a single i.v. injection of an AAV-CGN2-CAG-GFP vector. Animals were euthanized 3 weeks post-injection and tissues were harvested for downstream analyses. The efficiency and specificity of motor neuron transduction with the AAV-CGN2 vector were evaluated by immunofluorescence microscopy with native GFP fluorescence, pan-neuronal markers (NeuN, neuronal nuclei) and motor neuron markers (SMI-32 and choline acetyltransferase, ChAT). The AAV-CGN2 demonstrated a significant bias for transducing SMI-32-immunopositive upper motor neurons with minimal glia transduction. Notably, there were no significant differences in the specificity and efficiency of upper motor neuron transduction across the four species, reinforcing the strong upper motor neuron tropism of this capsid. As for the lower motor neuron transduction in the spinal cord, robust GFP signals were detected in cells with motor neuron morphology and in their axons all along the ventral horn in both cynomolgus and rhesus macaques. ChAT immunostaining revealed that the majority (>80%) of the lower motor neurons in the spinal cord were transduced with AAV-CGN2 in both macaque species. We are currently investigating spinal cord transduction profiles in marmoset. Preferred lower motor neuron transduction in the spinal cord was also evident in mice, albeit to a lesser extent compared to macaques, indicating potential interspecies variations. In summary, our study identified AAV-CGN2 as a highly potent upper and lower motor neuron-targeted capsid that demonstrates excellent cross-species compatibility. We envision that AAV-CGN2 vector will not only improve predictive modeling in preclinical studies but also enhance clinical translatability of AAV based gene therapies for MNDs.

P016

Machine learning-guided evolution of AAV capsids and synthetic promoters to enable systemically delivered gene therapies for the brain

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1: Shape Therapeutics

Adeno-associated virus (AAV)-delivered gene therapies hold great promise for treating central nervous system (CNS) diseases. However, the field is limited by wild-type AAV's inability to cross the blood-brain barrier (BBB), necessitating invasive methods of administration or very high doses leading to off-target transgene expression, particularly in the liver. Here we present complementary machine learning (ML)-guided capsid and promoter engineering platforms that overcome these challenges, promising to enable CNS-specific expression of a transgene at therapeutic levels delivered with intravenous AAV administration.

The AAVid capsid platform uses massive diversity capsid libraries and direct-to-non-human primate (NHP) biological selection to generate a high-resolution map of how AAV mutations affect capsid assembly and tissue tropism throughout the body. We first generated and screened a combinatorial library of over one billion capsid variants within the AAV5 sialic-acid binding pocket in adult cynomolgus macaques, recovering over 30 million unique capsid sequences from 50 tissues. To advance high-confidence capsid variants for the CNS, we developed novel computational pipelines for identification, ML generation, and functional comparison with wild-type AAV serotypes at the DNA and RNA levels. This ML-based selection and functional screening revealed CNS-targeting capsid variants with up to 1,000-fold enrichment in CNS targeting when compared to wild-type AAVs. We then performed singleplex validation of the highest-performing candidates, which confirmed greatly increased biodistribution in the NHP brain (~100x increase in brain and ~100x decrease in liver) following IV delivery compared to wild-type AAV9. These results indicate that the novel CNS targeting capsids can successfully deliver gene therapy payloads to the brain by IV administration while simultaneously minimizing off-target toxicity.

To further regulate the expression of the transgenes delivered by these CNS-targeting capsids, we developed a platform to generate strong and specific synthetic promoters tailored to the CNS. We employed Massively Parallel Reporter Assays (MPRAs) to screen over 80,000 promoters in a modular framework that combines a highly optimized core promoter with a cell-sensing enhancer, resulting in designs less than 216 bp in size. The libraries were screened in murine and human cell lines, with numerous promoters exhibiting high brain activity and minimal liver activity. Singleplex *in vivo* validation of the top promoters driving the expression of a progranulin (GRN) transgene yielded high quantities of GRN in cerebrospinal fluid and undetectable levels in serum, indicating greatly improved strength, specificity, and size profiles of the novel promoters compared to currently available options.

Our high-throughput, ML-guided platforms not only enabled the identification of CNS-targeting capsids and promoters but by their nature represent body-wide models of capsid and promoter sequence features, facilitating rapid application to other tissue expression profiles. Combining specific and tunable promoters with novel tissue-specific AAV capsids will bridge the gap between targeted delivery and tailored expression, accelerating the development of safe and effective gene therapies across the body.

P017

T-cell response to SRP-9001 dystrophin transgene in a patient treated with delandistrogene moxeparvovec: A case of immune-mediated myositis

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Delandistrogene moxeparvovec (SRP-9001) is an investigational rAAV vector-based gene therapy (GT), designed to compensate for missing functional dystrophin in Duchenne muscular dystrophy (DMD) by delivering a transgene encoding SRP-9001 dystrophin, an engineered dystrophin that retains key functional domains of the wild-type protein.

ENDEAVOR (NCT04626674) is an open-label, Phase 1b study assessing intended commercial process delandistrogene moxeparvovec material in patients with DMD. This study had a single case of immune-mediated myositis (IMM) in a patient with a deletion of exons 3–43 of the *DMD* gene. The patient experienced muscle weakness, with difficulties breathing and swallowing. The patient received immunosuppressive treatment, which included increased steroids followed by tacrolimus.

Clinical investigation with ELISpot analysis suggested that the IMM resulted from T cell-mediated responses directed against specific SRP-9001 dystrophin protein peptides. These peptides corresponded to exons 8 and 9, which encode the hinge 1 domain of dystrophin. *In silico* analysis of MHC presentation also suggested high immunogenicity of hinge 1 in this patient. Notably, hinge 1 is distinct from the spectrin-like repeats that form most of the dystrophin protein and is one of the least conserved regions compared with the closely related utrophin protein. We hypothesize that this combination of factors led to the lack of SRP-9001 protein immunologic tolerance and subsequent IMM in this patient.

These results are consistent with findings from clinical trials of other investigational micro-dystrophin GTs, which show that patients with deletions in regions of the *DMD* gene overlapping those expressed in a given micro-dystrophin may be at an increased risk of a myositis event following GT. Work is currently underway to better understand these risk factors and to find ways to safely administer delandistrogene moxeparvovec to patients with potentially higher-risk *DMD* mutations.

P018

Novel AAV capsids for the generation of chimeric antigen receptor-modified T-cells

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Modified cells, such as chimeric antigen receptor (CAR)-modified T-cells (CAR-T), are promising strategies for the development of novel therapies for malignant tumours. Conventionally, lenti/retroviral vectors have been used to semi-randomly insert the CAR coding sequence into the T-cell genome. While this strategy showed great success for many types of lymphomas, the resulting CAR-T product is characterised by high heterogeneity resultant from unique CAR expression profiles of each transgene integration site. This not only lowers the safety and potentially enhances CAR-T exhaustion, lowering the therapeutic efficacy.

To improve safety and efficacy, novel approaches to minimise clonal heterogeneity by utilising targeted gene editing mediated by clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 were applied. This approach, pioneered by Eyquem *et al*., 2017, used an adeno-associated viral (AAV) vector serotype-6 (AAV6) to deliver the CRISPR/Cas9 machinery and promoted homology-dependent repair (HDR)-mediated targeted insertion of a CAR into the *T-cell receptor alpha constant* (*trac*) locus. This strategy removed the safety and efficacy limitations associated with semi-random CAR construct integration and simultaneously knocked out the T-cell receptor, leading to further functional improvement of CAR-T cells.

While AAV6 used by Eyquem *et al* was successful, we hypothesised that the efficiency of the AAV-based CAR-T strategy could be further improved by utilising an AAV capsid that was developed for an optimal level of HDR-mediated CAR knock-in in T-cells.

We performed a selection of novel AAV capsids based on their ability to support on-target CRISPR/Cas9-mediated homology-directed knock-in into the *trac* locus in human T-cells. To do so, we developed and validated a proprietary AAV bioengineering platform that allows to select novel AAV variants based on their ability to mediate HDR-based editing at genomic locus of choice. We then utilised this platform to perform bio-panning of two peptide insertion libraries and one DNA family shuffled library on primary human T-cells and have selected 23 novel variants for further analysis based on deep-sequencing. The top enriched capsids were chimeric capsids containing regions of AAV4 and AAV6 as well as AAV6-peptide insertion capsids. After further testing and rational engineering of second generation candidates, which are chimeric and peptide modified, direct comparisons of homology-directed eGFP knock-in efficiencies into the *trac* locus were performed. The best performing novel candidate, named AAV-TC1 mediated ~50% knock-in with doses 1000-fold lower than the original study (1E6 vg/cell in Eyquem *et al.* vs 1E3 vg/cell in our study). Interestingly, the conventional transduction of T-cells was not improved by our news variants, indicating that they mediate improved HDR in a specific manner due to the HDR-driven selection technique.

To assess AAV-TC for its applicability in CAR-T therapies, the variant was used to insert a CAR targeting the erythropoietin-producing hepatocellular receptor tyrosine kinase class A2 (EphA2) into the *trac* locus in primary human T-cells. Using glioma as an exemplar of solid tumours, we found that CAR-T generated with our novel AAV-TC1 capsid showed high efficiency in tumour killing *in vitro*. The AAV-mediated EphA2-CAR-T approach is now evaluated in 3D- and brain-assembloid models of glioma for further validation.

P019

Rationally designed cardiotropic AAV2i8.I-1c demonstrates targeted cardiomyocyte distribution and a promising safety and efficacy profile in an ongoing phase 1 clinical gene therapy trial in patients with advanced heart failure

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Congestive heart failure (CHF) is a progressive disease in need of innovative therapies. A key characteristic of failing hearts is abnormal intracellular Ca^{2+} handling and increased protein phosphatase 1 activity (PP1). Recent advances in understanding the molecular basis of myocardial dysfunction and gene therapies have resulted in adeno-associated virus-based (AAV) gene therapy clinical trials for heart failure. Inhibition of PP1 by a constitutively active inhibitor-1 (I-1c) has been shown to enhance cardiac function in multiple pre-clinical models of heart failure. Earlier clinical trials with a cardiac gene therapy failed to show efficacy because of poor vector transduction at <0.012 vg/dg (vector genomes/diploid genome). We developed a novel cardiotropic and liver de-targeted capsid, AAV2i8, which was rationally designed to overcome poor transduction in cardiac tissue (1). Our ongoing phase 1 clinical trial for the treatment of non-ischemic cardiomyopathy uses the AAV2i8 capsid to deliver a constitutively active I-1c protein to cardiac tissue via a single intracoronary infusion of AAV2i8.I-1c. To date, eight patients have received either 3.25E13 (Cohort 1; n=3) or 1.08E14 (Cohort 2; n=5) viral genomes (vg) of AAV2i8.I-1c. There have been no investigational product–related serious adverse events through the 12-month observational period. Among participants in Cohort 1, all 3 showed clinically meaningful improvements in left ventricular ejection fraction (LVEF), New York Heart Association score (NYHA), Minnesota Living with Heart Failure Questionnaire (MLHFQ), cardiopulmonary exercise test (pV02 max), and 6 minute walk test (6MWT) at 12 months. Among participants in Cohort 2, 2 of 4 showed clinically meaningful improvements in MLHFQ, 2 of 4 showed clinically meaningful improvements in NYHA score, and all 4 showed clinically meaningful improvements in LVEF at 12 months when compared to baseline. In support of this encouraging early clinical efficacy signal, AAV2i8.I-1c demonstrated high myocardial transduction efficiency at 1.19 vg/dg in a human left ventricular (LV) sample taken 13 months post-intracoronary gene transfer from a patient during left ventricular assist device (LVAD) placement. The high transduction efficiency resulted in I-1c mRNA expressed at 26.7% of endogenous I-1 levels. We also observed a restoration of depressed S16 phosphorylation of phospholamban, which is associated with abnormal intracellular calcium handling, a key cellular abnormality in CHF. We subsequently developed methods to study our vector behavior at the cellular level in LV tissue sections. Vector genomes and I-1c mRNA were visualized in this LV tissue using *in-situ* hybridization (RNAscope) and cellular markers were visualized using immunofluorescence assays. I-1c mRNA was detected at high levels in cardiomyocytes but not in the endothelial cells in the biopsy sample, while control human heart tissue was negative for I-1c mRNA. Endogenous I-1 mRNA was detected in both the LV sample, and normal human hearts. Early results suggest that AAV2i8.I-1c (AB-1002) may provide encouraging safety and efficacy results in patients with non-ischemic CHF, which is further supported by emerging clinical biopsy biodistribution data.

References:

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P020

Preclinical evaluation of CAN201 containing a human *GLA* transgene under the control of a liver-specific promoter for Fabry disease

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Fabry disease (FD) is a rare X-linked lysosomal storage disorder caused by a deficiency of α-galactosidase A (α-GAL) as a result of mutations in the α-galactosidase A gene (*GLA*). The lysosomal accumulation of glycosphingolipids, especially globotriaosylceramide (Gb3) and globotriaosylsphingosine (lyso-Gb3), leads to a multisystemic disease, including kidney failure, myocardial infarction, and stroke. Enzyme replacement therapy (ERT) has been the standard of care for Fabry disease for two decades, but it is limited by incomplete treatment of disease manifestations, induction of anti-drug antibodies, and the requirement for lifelong biweekly infusions. Gene therapy offers potential advantages, including enhanced efficacy from increased enzyme exposure, reduced immunogenicity, and a single dose providing a durable response. We sought to develop an AAV gene therapy that would convert the liver into an in vivo manufacturing depot for α-GAL secretion into the circulation and uptake into other tissues. We screened vector constructs with different design elements for high protein expression, enzyme activity, and cell secretion in vitro. The lead clinical candidate, CAN201, contains an optimized enhancer, promoter, intron, human *GLA* transgene, and C-terminal element packaged into AAV.sL65, a human-liver-tropic vector.

Since AAV.sL65 does not efficiently transduce mouse liver, the construct was packaged into a surrogate mouse-liver-tropic vector, AAV.DJ (DJ-CAN201), to evaluate efficacy and safety in a Fabry mouse model. Fabry mice at 10-12 weeks of age were given a single intravenous (IV) dose of DJ-CAN201 at 1E11, 3E11, 1E12, or 3E12 vg/kg, and compared to vehicle-treated wild type or Fabry mice over 16-weeks. DJ-CAN201 treatment resulted in dose-dependent increases in liver CAN201 DNA and α-GAL mRNA levels, plasma α-GAL enzyme activity levels (14.8 to 1004-fold higher), and α-GAL enzyme activity levels in target tissues (liver, kidney, heart, spleen, skin, and brain). Concurrently, there were dose-dependent decreases in plasma lyso-Gb3 and tissue Gb3 levels. Plasma lyso-Gb3 was undetectable in animals at 3E12 vg/kg. Immunohistochemistry showed dose-dependent increases of α-GAL staining in tissues. DJ-CAN201 was well tolerated with no significant effects on body weight, tissue weights, or clinical chemistry; no macroscopic findings; and minimal microscopic findings.

The PXB mouse model containing a humanized liver was used to evaluate the transduction efficiency and expression of CAN201, the AAVsL65 clinical candidate. A single IV dose of CAN201 at 1E12, 3E12, 1E13, or 3E13 vg/kg resulted in dose-dependent increases in cell transduction (up to 9.6 vg/cell) and plasma α-GAL activity (up to 10.3-fold compared to vehicle). Target tissues (liver, kidney, heart, and spleen) also demonstrated dose-dependent increases in α-GAL activity (up to ~2 to 3-fold).

In conclusion, the DJ-CAN201 surrogate vector demonstrated efficacy and safety in Fabry mice, while in PXB mice, the CAN201 clinical candidate produced high circulating levels of α-GAL that were taken up by peripheral tissues. These preclinical studies support the further clinical development of CAN201 for Fabry disease.

P021

Next-generation sequencing guided AAV peptide library screening yields novel capsids for the central nervous system

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Gene therapy holds promise for treating numerous disorders, including neurodegenerative diseases. The development of CNS tropic engineered Adeno-associated virus (AAV) capsids to enable efficient delivery of therapeutic genes to cells within the central nervous system (CNS) remains a significant challenge.
Here we applied next-generation sequencing (NGS) guided screenings of 3 fully randomized CNS-tailored AAV peptide insertion libraries 1) 7-mer peptide design 2) 12- mer peptide design, and 3) liver-de-targeted 7-mer design. Following three rounds of iterative selection in mice, we performed an in depth bioinformatic analysis comparing CNS-derived peptides against sequences captured from various off-target tissues including liver and skeletal muscle as well as library intermediates to monitor and control the screening process. Finally, CNS captured sequences were ranked based on multiple criteria such as correlation pattern, peptide clustering, peptide abundances, enrichment scores (log2fold-changes) and specificity scores (k-scores).

Sequence analysis from CNS-captured AAV libraries revealed a strong enrichment of a set of unique peptide sequences and clustering for each of the three library designs. In addition, increasing the in-life phase strongly impacted the stringency of *in vivo* biopanning indicated by a time-dependent decrease of sequence correlation between on and off-target tissues suggesting higher CNS-specificity of the candidates.

Remarkably, candidates obtained from 12-mer insertion libraries and the liver-detargeted libraries outperformed candidates obtained from the heptamer libraries in terms of critical rankings suggesting performance with regards to CNS transduction efficacy and specificity. Finally, nominated candidates were successfully validated in a barcoded approach *in vivo* in mice. In line with the NGS results from the complex libraries, the barcoded approach confirmed enhanced neurotropism of various candidates, namely BI_R339_005 and BI-R339_006 that outperformed the current benchmark AAV as AAV9.PHP.eB in terms of transduction by a magnitude of ~3-5 folds respectively.

In conclusion, our study highlights the importance of optimizing peptide library design, fine tuning of the screening process, and stringent bioinformatic analysis driven candidate nomination to improve the development of novel efficient and specific AAV variants. Our data show that 12-mer insertions are potentially a critical factor that could enhance AAV neurotropism.

P022

Tumour-targeted DART-AAVs as gene delivery platform for immune-modulators

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In the context of cancer immunotherapy, successful administration of immunotherapeutic agents within the tumour microenvironment plays a pivotal role in maximizing therapeutic efficacy. Given the immunosuppressive characteristics of the tumour microenvironment, novel delivery approaches are currently being explored. Adeno-associated virus (AAV) vectors are the leading platform for *in vivo* gene delivery and have great potential to deliver immunotherapeutic to the tumour microenvironment. However, one of the crucial challenges associated with the delivery is off-target activities, resulting in unintended transduction of therapy-irrelevant tissues, most notably the liver. This phenomenon can lead to potential toxicity and significantly limit the applicability of the therapy.

To address this issue, we have developed a rational design strategy for AAVs based on display of high-affinity binders for a tumour surface antigen, such as the HER2/neu-specific designed ankyrin repeat protein (DARPin), at the VP2 N-terminus. We have recently shown that the resulting HER2-AAV enables tumour-specific delivery of checkpoint-inhibitors. In context of glioblastoma, this resulted in substantially improved therapeutic activity in a subcutaneous tumour mouse model when combined with CAR-mediated tumour cell killing (Strecker et al., 2022). Here, we generated and analysed a second-generation targeted platform called DART-AAV, in which DARPins are inserted into the VP1 GH2/GH3 loop. The corresponding second-generation HER2-AAV selectively transduced cancer cells only if they express human HER2/neu. Compared to the first generation, it exhibited a substantial increase in gene delivery activity of more than 20-fold without being compromised in selectivity. When equipped with the coding sequence for the checkpoint-inhibitor Nivolumab, transduced HER2-positive tumour cells released functional Nivolumab at high amounts *in vitro*. When administered into nude mice subcutaneously engrafted with HER2-positive SKOV-3 cells, HER2-AAV2 delivered luciferase specifically into the tumour tissue with the absence of detectable transduction in liver. As an additional tumour cell target, we have developed DART-AAV displaying DARPins specific for programmed death-ligand 1 (PDL1). PDL1-AAV demonstrated efficient transduction of PDL1-positive tumour cells, while PDL1-negative cells did not exhibit significant reporter gene expression. This additional example confirms the high selectivity of DART-AAVs for their target cells.

Ongoing investigations explore the extension of tumour-targeted DART-AAVs to other AAV serotypes as well as their therapeutic capacity in tumour mouse models. They appear especially suited for the delivery of immune checkpoint inhibitors and cytokines into the tumour microenvironment.

P023

BMN 293 – a novel gene therapy candidate for *MYBPC3*-deficient Hypertrophic Cardiomyopathy

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Hypertrophic cardiomyopathy (HCM) is a life-threatening inherited heart disease characterized by left ventricular hypertrophy (LVH) and diastolic dysfunction. The most common cause of HCM is variants in the *MYBPC3 gene*, which encodes cardiac myosin binding protein C (cMyBP-C), a sarcomeric protein with structural and regulatory roles. The majority of *MYBPC3* gene variants are truncating leading to protein haploinsufficiency. We have developed BMN 293, a novel adeno-associated virus (AAV) gene transfer vector for *MYBPC3*-deficient HCM that utilizes unique expression and regulatory elements to achieve cardiac selectivity and uniform distribution throughout the myocardium. During vector genome and production platform selection, multiple vector candidates generated in either the HEK293 mammalian or baculovirus insect production platforms were screened in *MYBPC3*-deficient human iPS-cardiomyocytes and mice; BMN 293 was selected based on having the highest expression and functionality in both systems. Interestingly, vector candidates produced in HEK293 cells consistently showed higher *MYBPC3* expression in both systems than vectors produced in insect cells. In long-term dose-ranging studies in *MYBPC3*-deficient mice, BMN 293 was well tolerated and resulted in high levels of human *MYBPC3* mRNA and cMyBP-C protein in sarcomeres throughout the heart and improvement of cardiac abnormalities, including contractile kinetics and LVH. Functional improvements were observed soon after dosing and remained durable throughout the study. BMN 293 intravenous administration to healthy primates at a high dose, without concomitant immunosuppression, was also well-tolerated and yielded uniform distribution of cMyBP-C throughout the heart. Together these results support further development of BMN 293 for *MYBPC3*-associated HCM.

P024

Rational Design Of Regulatable Expression Cassettes; Predicable, Robust, and Tuneable Expression in Non-Human Primates.

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In early gene therapy (GT) studies it was discovered that gene expression could cause toxicity at a cellular and systemic level. To combat this, innovations to improve the expression cassette - such as reducing CpG content, codon optimisation, reduction of viral components and tissue specific promoters - have been vital in enabling the development of safe and efficacious therapies. Despite these advances, most DNA-based GT rely on the use of constitutively active promoters which means there is no control over transgene expression levels. This can cause adverse outcomes in patients as overexpression or under expression of the transgene leads to toxicity or decreases the efficacy of the treatment. To address this problem, we describe the design and exemplification of a regulatable system in NHPs. The system was constructed using a bioinformatics pipeline which enables “to spec” design with background, expression strength and duration robustly and predictably conferred. Delivery via AAV, to either mice or NHPs, showed the system had no background expression and was dose responsive to a small inducer molecule. Indeed, the maximum induced levels were many-fold higher than currently used constitutive promoters. Furthermore, identical induction and expression kinetics were observed over multiple rounds of drug administration, with no difference between oral or IP delivery of the drug. We believe this system allows exquisite control of the gene of interest and may validate our pipeline for generating safer and more efficacious GT treatments.

P026

Searching the hairpin in the haystack: Tracing the impact of AAV-ITR mutations

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In Adeno-associated viral (AAV) vectors, engineering of the ITR (inverted terminal repeat, i.e., replication and packaging cis element) sequences may 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 toolbox for AAV ITR engineering which includes a vector construct design that allows easy modification of plasmid ITR sequences. This is complemented by a strategy for plasmid ITR sequence confirmation that can be used with conventional Sanger sequencing chemistry. To trace the ITR mutants during production and transduction, each ITR variant is associated with a specific DNA barcode in the 5‘ UTR of a transgene cassette on the virus genome. Titers of the resulting vectors based on this alternative design are similar to the traditional vector backbone. The integrity of the produced viral genomes and the presence of ITR mutants was examined by Nanopore sequencing. There, we discovered a previously undescribed and biologically highly interesting trans-acting ITR-repair mechanism in which ITR variants derived from different plasmids can serve as repair

template. Consequently, by avoiding the presence of different ITR variants during vector production, this ITR repair mechanism can be circumvented, permitting AAV vector generation with mutant ITRs that are maintained in the virus genome. Subsequent barcode interrogation by deep sequencing enables the determination of barcode distribution at high resolution, which, in turn, allows to quantify the impact of the mutations on AAV genome replication and packaging. Barcode sequencing in extracted RNA can also serve as a qualitative and quantitative measure for effects of the ITR on vector transduction efficiency, despite the potential loss of the ITR by recombination in the nucleus. Altogether, the novel pipeline for ITR modification and tracing reported here forms the basis for the comprehensive analysis of alternative ITR designs and their function during AAV production and transduction, which should ultimately benefit the creation and optimization of next-generation AAV vectors not only on the capsid but also on the genome level.

P027

Hepatoencephalopathy due to *GFM1* mutations: preclinical study of an AAV-based gene therapy in a mouse model of the disease

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The hepatoencephalopathy due to mutations in *GFM1*, also known as combined oxidative phosphorylation deficiency type I (COXPD1) is a recessive mitochondrial disease caused by mutations in the nuclear gene encoding the mitochondrial translation elongation factor G1 (EFG1), with no currently available cure. Patients with COXPD1 develop a severe encephalopathy, sometimes combined with liver failure, with neonatal onset and rapid progression that normally leads to death during the first weeks or years of life.
We previously obtained a genetically modified murine model of COXPD1 carrying a knockout allele and the p.R671C mutation, which has been found in at least 10 patients who survived more than 1 year, in the other allele (*Gfm1*$^{R671C/-}$) (Molina-Berenguer *et al*, FASEB J 2021). These mice show a clear dysfunctional molecular phenotype in liver and brain at 8 and 30 weeks of age: drastic EFG1 protein reduction causing impaired mitochondrial translation and combined OXPHOS deficiency (lower amount of assembled complexes I and IV causing a reduction of their enzymatic activities).

We have tested a gene therapy approach using an adeno-associated viral vector (rAAV) to introduce the correct copy of the human *GFM1* gene into *Gfm1*$^{R671C/-}$ mice. A single intravenous injection of an AAV9 vector carrying the human *GFM1* coding sequence under the control of the alpha-1-antitrypsin promoter preceded by an ApoE enhancer sequence (ssAAV9-hAAT-GFM1) on 6-week-old *Gfm1*$^{R671C/-}$ mice (5E+12 vg/kg), resulted in partial recovery of EFG1 levels in liver mitochondria four weeks after the treatment. Moreover, it entailed rescue of assembled complexes I and IV and their enzymatic activities in the target tissue, indicating that the transgenic product should be functional in mouse mitochondrial translation.

In conclusion, we have obtained preclinical evidence of efficacy for an AAV-mediated gene therapy for COXPD1, pointing to this strategy as a promising potential treatment for COXPD1.

P028

Optimized human genomic regulatory elements achieve DRG-selective de-targeting in non-human primates and reduce off-target toxicity in AAV gene therapies

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Adeno-associated virus (AAV)-mediated gene therapy has demonstrated transformative potential, but its application in the central nervous system (CNS) is limited by high transgene expression and toxicity in dorsal root ganglia (DRG). To address this challenge, we applied Encoded's next-generation sequencing and machine-learning platform to discover human genomic regulatory elements (REs) that significantly reduced DRG expression without affecting brain expression. Our GEN1 libraries tested over 10,000 REs, identifying 3' UTR sequences showing DRG-specific de-targeting in mice. Through multiplex validation of top elements, we next demonstrated strong correlation in de-targeting performance in non-human primates (NHPs). Incorporating the top candidate into the design of a gene replacement therapy candidate successfully mitigated protein overexpression in DRG. In NHPs, inclusion of this de-targeting element reduced transgene expression levels in DRG regions by greater than 10-fold, without reduction in brain expression, and was accompanied by improved sensory nerve functionality. Finally, using large-scale data from GEN1 libraries to train machine learning models, we identified improved genome-derived de-targeting sequences, enhancing *in vivo* performance in a GEN2 screen. Our approach efficiently identifies REs driving selective expression, potentially minimizing off-target toxicity in gene therapies irrespective of capsid, gene therapy modality, or administration route. This approach holds particular relevance for disorders requiring precise targeting and has been implemented across multiple RE types (enhancers, promoters, UTRs) and cell type targets to drive more refined GT expression profiles.

P029

Chemically surface-modified AAV6 vectors combined with nanoblades are a promising alternative for gene knock-in in HSCs

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Nanoblades, 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 HSCs thanks to their surface co-pseudotyping with baboon retroviral and VSVG envelope glycoproteins. Incubation of HSCs with rAAV6 vector containing two homologous arms to the Wiskott-Aldrich syndrome (WAS) locus flanking a GFP expression cassette together with nanoblades, resulted in up to 40 % of stable expression cassette knock-in into the WAS gene locus in HSC. However, high doses rAAV6 induced HSC cell death. Comparing rAAV6 with rAAV2 encoding the donor DNA, we demonstrated that at high doses, rAAV2 was much less toxic and gave higher transduction levels in HSCs. To improve donor template delivery, rAAV2 and rAAV6 were chemically bioconjugated with a mannose ligand, *via* the lysine or tyrosine amino-acid residues exposed at the capsid surface, in different quantities. Our results showed high level transduction of HSCs with mannose coupled rAAV6 vectors accompanied by a remarkable lower toxicity compared to the WT rAAV6. Mannose bioconjugated rAAV6 for DNA donor delivery combined with nanoblades allowed efficient gene knock-in and increased survival of HSCs from 40% to 80% as compared to the WT-rAAV6. 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 HSCs, an important feature for clinical translation of HSC-gene editing strategies.

P030

AVB-101 Six-month preclinical safety and biodistribution data following intrathalamic delivery to cynomolgus monkeys demonstrates good tolerability and widespread progranulin expression in brain tissue

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Frontotemporal dementia (FTD) is a devastating form of early-onset dementia characterised by a rapid decline in executive function, behaviour and/or language, typically leading to death within seven to 10 years of diagnosis. Mutations in the *GRN* gene are causative for a significant proportion of familial FTD cases (FTD-*GRN*) due to haploinsufficiency of progranulin (PGRN). There are currently no disease-modifying treatments for FTD-*GRN*. AVB-101 is an AAV9 gene

therapy that drives the expression of human PGRN (hPGRN) under the control of a neuronal-specific promoter and is being developed to treat FTD-*GRN*. AVB-101 aims to restore physiological levels of PGRN in the central nervous system of FTD-*GRN* patients. Previously, we reported that low doses of AVB-101 can rescue pathology in the *Grn* knock-out mouse model when administered into the thalamus. To evaluate the safety and biodistribution of AVB-101, a GLP toxicology and a companion biodistribution study were conducted in cynomolgus monkeys. Animals received a one-time bilateral convection-enhanced infusion of AVB-101 into the thalamus and were monitored for up to six months. Both the low- and high-dose of AVB-101 tested were well tolerated, with no mortality or clinically evident adverse effects. Biodistribution analysis showed that hPGRN was most abundant in the thalamus but detected throughout the brain. Physiological levels were reached in the temporal and frontal lobes, which are the cortical regions most severely affected in FTD-*GRN*. AVB-101 vector was minimal or undetectable in most visceral tissues, and hPGRN expression was restricted to the central nervous system (CNS). Levels of progranulin in the cerebrospinal fluid showed a dose-dependent increase, offering a potential biomarker of vector transduction and expression in the CNS. Taken together, the non-clinical data on AVB-101 is supportive of progressing to human clinical trials.

P031

AAVrh.10 delivery of the combined E2 and Christchurch gain-of-function variants of the human APOE gene effectively suppresses both amyloid and Tau pathology in the CNS of murine models of APOE4 homozygote Alzheimer's Disease

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Gene therapy to treat hereditary disorders conventionally delivers the normal allele to compensate for the loss-of-function caused by the inherited mutation. There are rare examples, however, when more effective gene therapy can be achieved using a gain-of-function human variant, such as the Padua variant used to treat factor IX deficiency, resulting in a marked improvement in efficacy for the same dose compared to using the normal allele. The focus of this study is to assess combining the rare human APOE Christchurch (R136S) APOE variant with the common human APOE2 (C112/C1558) variant to generate a highly effective gain-of-function variant to suppresses both the amyloid and tau-associated pathology in murine models of APOE4 homozygote Alzheimer's disease (AD). We tested the hypothesis that AAVrh.10-mediated CNS delivery of the combined human APOE2 allele with the Christchurch mutation (AAVrh.10hAPOE2Ch, referred to as "E2Ch"), will provide superior protection against development of AD-related pathology compared to the unmodified APOE2 allele (AAVrh.10hAPOE2, referred to as "E2"). These vectors along with controls AAVrh.10Null and PBS were tested in two models of AD: APP.PSEN1/TRE4 "amyloid mice" that develop amyloid plaques with progressive neurodegeneration; and P301S/TRE4, "tau mice" that develop neurofibrillary tangles and tauopathy with progressive neurodegeneration. The vectors or controls were administered to the hippocampus ($2x10^{10}$ gc, 2 µl) of amyloid mice at age 2.5 months with assessment 3 months post-administration and to tau mice at age 5.5 months with assessment after 3 months. Both the E2Ch and E2 vectors prevented Aβ42 and Aβ40 accumulation in amyloid mice compared to controls ($p<0.01$) but only the E2Ch vector suppressed total tau and p-tau levels in tau ($p<0.01$) mice. Both the E2Ch and E2 vectors decreased β-amyloid aggregates in

amyloid mice but a decrease in tau tangles in tau mice was observed only with the E2Ch vector ($p<0.01$). Both the E2Ch and E2 vectors inhibited neurodegeneration (Fluro Jade staining) of amyloid mice ($p<0.01$), but only the E2Ch vector inhibited this in tau mice ($p<0.01$). Microglial activation (Iba1 staining) and reactive astrocytes (GFAP staining) were significantly suppressed with both vectors in amyloid mice ($p<0.01$), but only the E2Ch vector mediated significant suppression of Iba1 and GFAP in tau mice ($p<0.01$). Lastly, animals were assessed using behavioral assays (nesting, Y maze, novel object recognition, Barnes maze). In the amyloid mice, the E2 and E2Ch vectors had similar function, but in the tau mice, the E2Ch vector outperformed the E2 vector, with E2Ch improvement in all 4 behavioral assays compared to E2. In summary, while the E2 vector is effective, the combined Christchurch and APOE2 variant is a “hyper” gain-of-function APOE variant that more effectively treats both the amyloid and tau pathology of murine models of APOE4 homozygous Alzheimer’s disease.

P032

In vivo-directed evolution discovers two novel AAVs targeting the glomerular endothelium and the juxtaglomerular apparatus in the kidney

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Kidney diseases represent a major and steadily increasing global health burden. Since kidneys are being composed of a large number of different cell types, assembled in various tissues with highly diverging functions and disease susceptibilities, cell-specific targeting strategies are urgently needed. Adeno-associated virus (AAV) as a promising *in vivo* delivery platform shows the advantage in delivering therapeutic molecules to those difficult or non-druggable cells. However, natural AAV serotypes have insufficient targeting specificity and transduction efficiency in kidney cells, thus approaches to broaden the tropism of AAV and screenings for kidney-specific AAV vectors are required. In this study, we aimed to discover new AAV vectors targeting different kidney cells. We developed a selection protocol specifically for kidneys and screened random AAV2 and AAV9 display peptide libraries *in vivo*. Integrative experimental and bioinformatics workflows were conducted to identify the most promising AAV vectors with high targeting specificity and transduction efficiency. The selected AAV vectors were evaluated among rodents under both physiological and pathological conditions. We identified two AAV variants termed AAV2-GEC and AAV9-JGA, which specifically and efficiently transduced the glomerular endothelium (GEC) and a subset of the distal tubule epithelium in the kidney, respectively. AAV2-GEC exhibited robust GEC tropism in C57BL/6J, Balb/c mice and Sprague Dawley (SD) rats, as well as in disease models causing GEC damage. The potential of AAV2-GEC for kidney-targeting therapy was evaluated by delivering to the GEC an antibody-cleaving enzyme, which successfully eliminated the kidney-bound IgG, thereby prevented the progression of glomerulonephritis. AAV9-JGA transduced the CLDN16/SLC12A1-positive cells of the thick ascending limb (TAL) at the juxtaglomerular apparatus (JGA) and maintained selective tropism in C57BL/6J mice and SD rats. We demonstrated that AAV9-JGA reached the TAL through the tubule-afferent arteriole contact after systemic administration, indicating that AAVs can directly reach the tubular segments from the bloodstream without crossing the glomerular filtration barrier. In conclusion, we developed a comprehensive strategy and workflow to screen AAV library in vivo and discovered two new AAV vectors that targeted the most important structures in the kidney. We revealed a natural AAV transduction route in the kidney after systemic

administration. This study identified the targeting of highly specific kidney cells demonstrating the feasibility of future cell-specific kidney therapies.

P033

A rational capsid-engineering strategy reduces innate and adaptive immune responses to Adeno-Associated Virus (AAV) Vectors

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Immune responses towards the AAV capsids or encoded transgene products pose a challenge for Adeno-Associated Virus (AAV) vector-based gene therapies. With the aim of reducing innate immune responses elicited upon vector administration, we engineered the capsid by inserting an immune modulatory peptide aiming to interfere with innate immune signaling upon cell entry. Insertion of this peptide did neither affect capsid assembly nor the empty-to-full capsid ratio or production efficiency. The new capsid variant, termed AAV2.MB, showed enhanced transduction efficacy in primary human cells including monocyte-derived dendritic cells (moDCs) and - in line with our hypothesis - a reduced innate immune response. Importantly, AAV2.MB vector administration either intravenously or intramuscularly resulted in a significantly reduced CD8+ T cell response against the AAV vector-encoded EGFP transgene product. Also, a reduced CD8+ T cell response against the capsid was observed in mice receiving the AAV2.MB vector intramuscularly. Beyond that, humoral responses against AAV capsids were mitigated in AAV2.MB-injected mice independent of the route of administration. Thus, applying a rational capsid-engineering strategy we could modify the activation of innate as well as adaptive immunity in response to AAV2 vectors.

P034

Early liver transcriptomics of AAV5 gene therapy–treated haemophilia A dogs

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Recombinant adeno-associated virus vectors (rAAVs) are a major gene therapy platform for treatment of monogenic disorders, including haemophilia A. Valoctocogene roxaparvovec (AAV5-HLP-hFVIII-SQ) is an adeno-associated virus serotype 5 (AAV5) vector delivering B-domain–deleted (BDD) human FVIII transgene controlled by a hybrid liver-selective promoter.

In a phase 3 trial, a single treatment of AAV5-HLP-hFVIII-SQ ($6x10^{13}$ vg/kg) provided therapeutic expression of FVIII and bleeding control in adults with severe haemophilia A. However, the mechanistic bases of transaminitis, variability, and durability observed in clinical trial participants are not clear. Understanding the molecular changes in the liver are critical to identify appropriate immune-modulatory strategies for safety, efficacy, and long-term durability of AAV gene therapy. To address this, we investigated liver gene expression profiles before and after AAV5-HLP-canine-BDD-FVIII (cFVIII) administration in a severe haemophilia A dog model. Nine severe haemophilia A dogs received 1 of 3 vectors (non–codon-optimized AAV5-HLP-cFVIII-SQ, codon-optimized AAV5-HLP-cFVIII-SQ, and AAV5-HLP-cFVIII-V3 at $6.0x10^{13}$ to $2.0x10^{14}$ vg/kg). Liver biopsies were collected at baseline and 3 months after vector administration. FVIII activity was measured by one-stage FVIII assay using a pooled normal canine plasma standard. Liver cFVIII DNA and RNA levels were quantified using droplet digital PCR. Transcriptomic profiling was performed by RNA sequencing followed by pathway enrichment analysis. Cellular immune response in peripheral blood mononuclear cells (PBMCs) was evaluated using an interferon (IFN)-γ enzyme-linked immunosorbent spot assay. Dose-related FVIII expression was observed in dogs treated with the codon-optimized vectors, with significant correlation between liver vector DNA and circulating FVIII activity. Dogs treated with codon-optimized cFVIII vectors demonstrated enhanced transgene expression compared to the non–codon-optimized vector. No IFN-γ response was detected in PBMCs. Transcriptomic profiling of liver biopsies demonstrated enrichment of integrin pathways, immunological gene signatures for B cells and plasmacytoid dendritic cells, and common dendritic cells at 3 months compared to baseline. Expression of inflammatory cytokines involved in natural killer (NK)–cell and T-cell activation was also enriched. Our data suggest that mild activation of B cells, dendritic cells, NK cells, and T cells with an inflammatory cytokine response occurred in the liver of AAV5-HLP-cFVIII–treated dogs 3 months after gene transfer, albeit without transaminitis. Transcriptomic profiling of PBMCs is ongoing to compare to liver profiles to better understand kinetics of immune responses to rAAVs.

P035

Improvement of progressive familial intrahepatic cholestasis type 2 via AAV-mediated hepatic BSEP expression in mice

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Progressive familial intrahepatic cholestasis type 2 (PFIC-2) is a rare autosomal recessive disorder caused by mutations in *ABCB11* gene encoding for the liver bile salt export pump (BSEP), responsible for the transport of bile salts (BS) from hepatocytes to the canalicular lumen. BSEP absence or dysfunction results in impaired BS secretion and its accumulation in hepatocytes leading to severe liver damage. PFIC2 patients usually develop symptoms during infancy, including cholestasis, failure to thrive, jaundice, hepatomegaly, and severe pruritus. Most develop early fibrosis and end-stage liver disease and have an increased risk of developing hepatocellular carcinoma. Therapeutic approaches include pharmacological management of symptoms, surgical biliary diversion or ultimately liver transplantation, which currently is the only curative option. Adeno associated virus (AAV)-based gene therapy targeting the liver could represent a safe and efficient option for PFIC2 patients by restoring long-term hepatic BSEP expression and physiological bile secretion.

We first generated and characterized an *Abcb11*$^{-/-}$ mouse model of PFIC2 in a pure C57BL/6 genetic background. Bile salt secretion was dramatically impaired in both sexes with reduced BS levels in the bile and small intestine. Female mice showed a progressive elevation of serum transaminases and bilirubin levels starting at two months of age, while male mice only showed an increase in these biomarkers after six months of age. Hepatomegaly was observed in both genders but was significantly higher in females. Overall, females presented a more severe phenotype and recapitulated the human disease more closely than males.

We next assessed the therapeutic efficacy of gene therapy in this PFIC2 mouse model using a liver-tropic AAV8 vector carrying a codon-optimized human BSEP cDNA under the control of a liver-specific promoter (VTX-802(8)). Five-week-old PFIC2 female mice were injected with a single intravenous administration of VTX-802(8) at two different doses and followed until four months of age. Correct localization of BSEP in biliary canaliculi was observed in treated mice after sacrifice at either dose. Animals treated with the higher dose showed normalization of serum transaminases and bilirubin levels at three weeks post-injection. Moreover, these mice showed a partial but significant reversion of hepatomegaly, and the release of BS from the liver to bile and small intestine was significantly increased at 4- and 11-weeks post-injection, indicating that physiological bile secretion was partially restored.

These early results indicate that VTX-802(8) has therapeutic potential for PFIC2. However, further development of the AAV vector and/or combination with other pharmacological products, might be investigated to enhance treatment efficacy and further revert the disease biomarkers. The development of a durable cure for PFIC2 and its translation to the clinic would provide a life-changing alternative for these pediatric patients with high unmet medical need.

P036

Improving AAV packaging capacity by enhanced protein trans-splicing at low dual vector doses

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Adeno-associated viral (AAV) vectors represent one of the leading platforms for gene delivery. Despite significant improvements since their development, AAV vectors still present several limitations. One major bottleneck is the small packaging size restricting its use to treat diseases requiring the delivery of large genes. To overcome this, dual AAV vector delivery systems relying on homologous recombination have been explored to reconstitute oversize transgenes. Alternatively, protein trans-splicing systems can be used to reconstitute large proteins, being the split-intein Npu DnaE sequence from *Nostoc Punctiforme* currently the most used. However, it presents low reconstitution efficiencies, demanding higher vector dose administration to attain efficient therapeutic effects. Low-quality vector preparations can also contribute to limited gene reconstitutions by promoting vector competition and inhibiting transduction. This work overcomes inefficient protein reconstitution rates and decreases vector doses of current dual AAV vector-intein-mediated systems by applying split-inteins with unmatched trans-splicing rates and using high-quality AAV vectors.

Studies were conducted evaluating two split-inteins, engineered-consensus (Cfa) and cyanophage-like (Gp41-1), with reportedly superior trans-splicing in comparison with Npu DnaE

split-intein. Protein reconstitution was assessed by transient transfection and dual-AAV *in vitro* co-transduction. The impact of empty vector particles in the co-transduction efficiencies and in split-inteins' performances was also evaluated. An upstream-to-downstream process was performed with and without a full genome particle enrichment step (using anion exchange chromatography). Two different sets of AAV2 vector quality preparations for each split-intein under evaluation were obtained.

The assessment of the split-inteins' protein reconstitution efficiencies revealed that both Cfa and Gp41-1 split-inteins enabled reconstitution rates that were over 2-fold higher than Npu DnaE and showed 100% of protein reconstitution. The established AAV vector full particle enrichment step increased the quality of the viral preparations by 3-fold, as lower quality preparations presented an average of 20–30% of full AAV particles and higher-quality preparations (subjected to an additional step of full genome particles' enrichment) an average of 60–75% of full AAV2. Higher-quality preparations increased split-inteins' performances by 3-fold when compared to low-quality preparations. Low-quality vector preparations were observed to limit split-gene reconstitutions by inhibiting co-transduction. Moreover, we show that combining superior split-inteins with higher-quality vector preparations allowed to decrease vector doses by 50-fold while maintaining exceptional trans-splicing rates. The results obtained establish the cornerstones for ongoing studies using therapeutic genes.

This work shows that efficient dual-AAV vector delivery combined with enhanced protein trans-splicing enables the efficient transfer of larger therapeutic genes, ultimately extending the clinical use of AAV vectors in gene therapies.

P037

Development of a scalable upstream platform process with 1E15 vg/L bioreactor titer for AAV

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High productivity and product quality have been crucial in developing novel AAV-based drug candidates, not only to fuel clinical trials, but also to reduce the high manufacturing cost per patient dose. The pursuit of high AAV productivity has led to an increased focus on the upstream processing. To improve bioreactor titer, OXB Solutions has developed a new-and-improved upstream process showing a 10-fold increase in bioreactor productivity, a 2-fold increase in percent full capsids, and consistent yields exceeding 1E15 AAV vector genomes (vg) per L of culture. This new platform has resulted in a 90% decrease in manufacturing cost per individual dose, which will prove essential to meet high patient demands.

Process improvements leading to the large increase in titers were the result of a combination of factors across the upstream process, all while maintaining, or even improving, the high level of product quality. Raw material evaluations were performed across a wide range of media, transfection reagents, and post-transfection additives. These evaluations confirmed the high performance of OXB Solutions process reagents and surprisingly identified an additive that resulted in up to a 3-fold titer increase. Integration of a dual-plasmid transfection system resulted in another 2-fold increases in vg titer and consistently higher percent full capsids. Additionally, the team overcame challenges around increased transfection cell density to successfully move from 2E6 to 4E6 cells/mL which led to an additional nearly 2-fold titer increase. Lastly, the team focused on bioreactor parameters for a further boost in productivity. This new high-performance

upstream platform has proven to produce bioreactor titers close to or above 1E15 vg/L and, in most cases, over 50% full capsids in affinity-purified product across all tested constructs, including 9 different AAV serotypes spanning 4 different Clades, as well as 8 different genes of interest (GOIs).

Another common challenge in the field of AAV process development is demonstrating scalability and reproducibility. The OXB Solutions upstream process delivers a robust and desirable product quality profile across manufacturing-friendly operating ranges. The new upstream process has demonstrated comparable titers and consistent product quality across multiple batches at 2L, 50L, and 500L scale for different constructs and AAV capsid serotypes. Successful scale-up of this process is enabled by critical control of our high-performing transient transfection.

The benefits of a highly productive upstream process may not be realized through to drug substance without a similar effort and focus on the downstream side. At OXB Solutions, we have combined our new 1E15 vg/L upstream process with a comparably intensified downstream process that allows us to manufacture over 1E17 vector genomes of drug substance per 500L bioreactor.

P038

Dyno-86m and Dyno-gvk: cell-type resolved validation of AAV capsids optimized for intravitreal delivery to the non-human primate retina

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Safer and more effective intravitreally delivered ocular gene therapies may be enabled by engineering AAV capsids for highly efficient transduction of the retina at low doses, thereby reaching a sufficient number of cells without eliciting an inflammatory response. Here we characterize Dyno-86m, a capsid with field-leading potential for intravitreal delivery. The Dyno-86m capsid was designed with generative AI leveraging in vivo data from multiplexed measurements of capsid libraries. In pooled experiments using NGS to measure bulk retina transduction in Cynomolgus monkeys, Dyno-86m was 80-fold improved vs AAV2.

Understanding delivery efficiency in specific cell types is key to successful ocular gene therapy. Towards this goal we further characterized the performance of Dyno-86m through single-nuclei RNA sequencing and histology, comparing to both wild-type AAV2 and an external engineered capsid.

We compared Dyno-86m to the external engineered capsid in a 2-capsid Cynomolgus monkey study, co-injecting each at a dose of ~1e11vg per eye. Transduction was measured through cell-segmentation and quantification of immunofluorescent histological images. Dyno-86m consistently outperformed the external engineered capsid, with 2.6-fold higher transduction across the entire retina, and between 2.2 and 3.2-fold higher transduction across ganglion cells, inner nuclear layer cells including bipolar cells, and photoreceptors in the central retina.

In a separate Cynomolgus monkey study, single-nuclei RNA sequencing confirmed Dyno-86m delivery to all major NHP retinal cell types, including ganglion cells, rods and cones. Dyno-86m showed 2-3 fold higher transduction across these cell types compared to the external engineered

capsid, closely matching histology results from the 2-capsid study. Similar results were observed when quantifying transduction of the macula by snRNA-seq, where percent cells transduced was greatest.

These results demonstrate the potential of Dyno-86m as the field-leading capsid for intravitreally delivered gene therapies, opening new opportunities for gene therapies to treat a wider range of ocular diseases.

P039

ICG-mediated ILM photodisruption enhances AAV transduction following intravitreal injection in rabbits

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The inner limiting membrane (ILM) represents a major bottleneck for the retinal delivery of therapeutics following intravitreal injection. Overcoming the ILM barrier could hence signify a great leap forward for many retinal therapies in the pipeline. Making use of the clinically approved photosensitizer indocyanine green (ICG) and pulsed laser light, we aim to photoporate the ILM in a safe way, allowing therapeutics to enter the retina in a highly efficient manner. Following up on our proof-of-concept study in bovine explants, this *in vivo* study further explores the safety and efficacy of ICG-mediated ILM photodisruption.

3 New Zealand White rabbits (6 eyes, age 3–6 months) received ILM photodisruption treatment. First, 40 µl of 0.625 mg/mL ICG solution was injected into the vitreous close to the retina to maximize ICG binding to the ILM. To allow unbound ICG to be cleared, laser treatment was performed 4 days following ICG injection. To this end, anesthetized rabbits were placed on a stabilization platform after which a 5 by 5 mm2 area per eye was irradiated with laser pulses (<7 ns, 800 nm) at a fluence of 1.9 J/cm^2. After intravitreal injection of AAV9 vectors (1x10^{12} vg/mL) on the same day, animals were monitored for 4 weeks with Photoacoustic imaging, optical coherence tomography (OCGT), color fundus photography, fluorescence imaging (FI), and electroretinogram (ERG). 1 rabbit which received an AAV9 injection without ILM photodisruption treatment served as a control.

Based on the FI imaging, we demonstrated that for all 6 eyes AAV9 transduction in the laser-treated area was substantially higher. Fluorescence intensity measurements of the treated area revealed a 2.4 fold increase at day 7 as compared to the control animal, a trend which persisted for the next 3 weeks. OCT, dark-adapted and light-adapted ERG did not reveal any effect on retinal morphology or function, respectively.

This study demonstrates that ICG-mediated ILM photoporation is a safe method to overcome the ILM and enhance retinal therapeutic delivery. This technology is able to greatly promote the delivery and hence transduction potency of viral vectors into the retina following intravitreal injection. Driven by this positive outcome, future plans include further *in vivo* exploration of its potential for other therapeutic classes including nanoparticles and cells.

P040

Airway-specific gene expression and biodistribution in ferrets and nonhuman primates utilizing recombinant Human Bocavirus Type 1

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The lung presents several obstacles to gene delivery that provides selective and durable gene expression, prevents achieving meaningful clinical outcomes for severe pulmonary diseases, including cystic fibrosis (CF). Current viral-based gene delivery approaches are limited by genetic capacity and nonspecific tropism for lung epithelial cells. Human Bocavirus Type 1 (HBoV1) is a nonpathogenic parvovirus of genus *Bocaparvovirus* that readily infects human airway epithelial cells repeatedly and offers 20% extra genomic capacity beyond adeno-associated virus 2 (AAV2). CBN-1000 is a hybrid viral vector featuring a HBoV1 capsid and engineered with conventional AAV2-vector genome elements. The hybrid vector displays exquisite lung tropism while simultaneously leveraging the much of the safety, regulatory and CMC profile of AAV vectors. In the present study, we evaluated the cell type transduction and durability of CBN-1000 in primary human lung epithelial cells from normal donors as well as those from CF patients cultured in air-liquid interphase and differentiated into mature human airway epithelial cells that resemble airway epithelium *in situ*. Results indicate sustained transduction of both progenitor basal cells and terminally differentiated specialized cells of the airway epithelium.

Studies in ferrets and nonhuman primates were conducted to characterize the biodistribution and expression of a GFP transgene delivered by CBN-1000. In addition, an ^{124}I-labeled HBoV1 capsid was administered intratracheally and evaluated for tissue tropism in wild-type (WT) and CF transgenic ferrets. Results indicate similar biodistribution, tissue penetration and clearance in both WT and CF ferrets, despite a moderate-to-severe lung disease phenotype in the transgenic animals. In addition, selective biodistribution and gene expression in the lung was demonstrated in both ferrets and nonhuman primates. Administration of CBN-1000 was well tolerated in both species at clinically relevant dose levels. Overall, these data suggest CBN-1000 is a novel and selective viral vector for lung delivery with the potential for delivering transgenes up to 5.5kb in healthy or diseased lungs resulting in durably expression. CBN-1000 solves several of the most daunting challenges of current pulmonary gene delivery and exemplifies the potential of capsids derived from non-AAV, autonomous parvoviruses to expand the scope of gene therapy for diverse applications.

P041

Characterization of AAV integrations and rearrangements from long and short reads with RAAVioli

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Gene therapy (GT) applications based on recombinant Adeno Associated Viral (rAAV) vectors were proven successful in treating several diseases, thanks to their high transduction efficiency and specificity. Even though rAAV primarily remains episomal in the nucleus of transduced cells, a growing number of studies published over the past years have demonstrated the integration of fragmented or full-length AAV DNA within the transduced cell genome where double-strand DNA breaks (DSBs) or nicks have occurred. Yet, the occurrence of hepatocellular carcinoma and clonal expansion events consequent to rAAV insertions have posed safety concerns for their clinical use.

However, bioinformatics tools able to identify AAV integration sites (IS) and characterize vector rearrangements are still missing. Here, we studied the DNA collected from a humanized liver mouse model, where human primary hepatocytes have been transduced ex-vivo or in-vivo with a tomato expressing AAV. AAV IS were retrieved using SLiM-PCR and short-paired end Illumina technology as well as by long reads PacBio sequencing after probe-based capture. Sequencing reads were analysed using RAAVioli (Recombinant Adeno-Associated Viral IntegratiOn analysis) to characterize vector rearrangements and IS. Python and R scripts parse the alignments to identify IS and reconstruct rearrangements using CIGAR strings.

Overall, 811 and 370 unique IS were identified from short paired-end Illumina reads and long PacBio reads respectively, confirming the higher efficiency of PCR-based approach. The IS were distributed all along the human genome showing the typical preference of targeting CpG islands and transcriptional start sites. Moreover, 32 IS were found in both datasets demonstrating the consistency of the results obtained independently from the platform adopted. Both IS datasets showed a similar percentage (~25%) of fragments containing AAV rearrangements, although more than 2 and up to 6 AAV rearrangements were observed only when long PacBio reads were adopted.

Precision and accuracy of RAAVioli pipeline were assessed through simulated datasets obtaining scores >0.95 in IS identification and rearrangement characterization.
These results indicate that RAAVioli is a versatile and comprehensive bioinformatic tool that can efficiently map AAV IS using both long and short paired ends sequencing reads. These approaches are fundamental to characterize AAV integration and recombination events in gene therapy and gene editing applications, allowing and improving the assessment of safety in AAV studies.

P042

Tracking of AAV5 vector integrations and clonal expansion in mice long-term

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Recombinant adeno-associated virus (rAAV)–based vectors are used clinically for gene transfer for their ability to effectively transduce human cells. Most rAAV vector genomes persist in cells as extrachromosomal episomes. Vector genomes can integrate into the host genome, but the theoretical risk of tumorigenesis may depend on vector regulatory features. We used a mouse model to investigate the long-term kinetics and integration profiles of an rAAV serotype 5 (rAAV5) vector that mimics key features of valoctocogene roxaparvovec (AAV5-hFVIII-SQ), a gene therapy for severe haemophilia A. We used an rAAV5-human alpha-1 anti-trypsin (hA1AT) vector approximately the same size (~5 kb) and containing the same regulatory elements as valoctocogene roxaparvovec. Mice do not develop antibodies to hA1AT protein, thereby allowing long-term studies. C57BL/6J mice received $6x10^{13}$ vg/kg of vector produced in insect *Spodoptera frugiperda* (*Sf*) and HEK293 cells. Mice were sacrificed at weeks 1, 3, 12, 24, and 57, and DNA and RNA were extracted from their liver samples. Target enrichment sequencing, common integration site (CIS) analysis, and polyclonal-monoclonal distance (PMD) tools were used to characterize vector integration profiles over time. The majority (88.7%–97.4%) of vector genome reads retrieved from *Sf* vector–treated mice contained vector-vector junctions, with high representation of inverted terminal repeats, representing the episomal form of the vector. The average vector integration frequency was 2.70 (standard deviation, 1.24) integrations per 1000 cells, and remained constant up to 57 weeks post-dose. To determine if integration sites (IS) accumulate at specific genomic regions, CIS analysis was performed, which attributes a CIS order based on the number of unique integrations spanning a CIS region. In samples from *Sf* vector–treated mice, 11.7% of CISs had an order ≥5, suggesting a proportion of these integrations occurred in a non-random manner. Integrations were enriched near the transcription start sites of genes highly expressed in the liver ($P = 1x10^{-4}$), and less enriched for groups of genes with low or no liver expression. We used a PMD index tool to evaluate the diversity of IS across samples. This tool estimates clonality by measuring the distance between richness, represented by the number of IS within a sample, and evenness, represented by the relative frequencies of each IS.
The *Sf* vector–treated samples clustered near the theoretical maximum for polyclonality, indicating a lack of clonal expansion throughout the study. This provides molecular support for the absence of tumours observed by histology in vector-treated mice. Results from mice treated with HEK293-produced vector confirmed those from *Sf*-produced vector. Our longitudinal integration analysis suggests the AAV5-hFVIII-SQ integrations occur within 1 week at a low frequency and do not increase with time. Most importantly, we found no evidence of clonal expansion at a molecular or histological level.

P043

New AAV9 engineered variants with enhanced neurotropism and reduced liver off-targeting in mice and marmosets

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Adeno-Associated Virus 9 (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. We generated a new peptide display library starting from a galactose binding-deficient AAV9 capsid and selected two new AAV9 engineered capsids, named AAV-Se1 and AAV-Se2, with an enhanced targeting in mouse and marmoset brains after intravenous delivery. Interestingly, the loss of the galactose binding strongly reduced the undesired targeting to peripheral organs, and above all liver, while not compromising the transduction of the brain vasculature. However, we had to reconstitute the galactose binding in order to efficiently infect non-endothelial brain cells. Thus, the combinatorial actions of the galactose-binding domain and the installed exogenous displayed peptide are crucial to enhance BBB crossing together with brain cell transduction. We also identified Ly6C1 as primary receptor for AAV-Se2 which is a Ly6A homologue highly expressed in the brain endothelial cells. This study describes a new strategy to select neurotropic AAV9 variants and identifies two novel capsids with high brain endothelial infectivity and extremely low liver targeting based on manipulating the AAV9 galactose binding domain.

P044

AAV vector-based transgene expression in primary human NK cells

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Natural killer (NK) cells are specialized innate immune effector cells. Their innate ability to recognize and kill tumorigenic cells makes them an interesting candidate for cellular immunotherapy, especially in combination with chimeric antigen receptors (CARs). Indeed, CAR-NK cells are considered as an alternative approach to CAR T cells with the advantages of a better safety profile with no or minimal neurotoxicity or cytokine release syndrome and the potential for an allogenic “off-the-shelf” manufacturing. However, the safe and efficient modification of NK cells using viral or non-viral vector gene transfer is still a challenge.

Here, we report on establishing adeno-associated virus (AAV) vectors as a novel and promising gene transfer tool for NK cells. We systematically tested a broad range of AAV vectors for uptake and transgene expression in human peripheral blood mononuclear cell (PBMC)-derived NK cells. We determined the impact of in vitro culture conditions, cultivation times, as well as different transduction protocols on transgene expression.

The level of transgene expression did not seem to be linked to cell entry but rather to the NK activation state and to cellular metabolic processes. In general, the basal level of transgene expression was highly donor dependent, ranging from below 10 % up to more than 50 %. By optimizing transduction conditions, we now reach a donor-independent increase of the eGFP transgene expression levels to around 80%.

In conclusion, we report here for the first time efficient AAV vector-mediated transgene expression in primary human NK cells. Our optimized conditions for ex vivo NK cell transduction, are now used to equip NK cells with anti-CD19 and anti-CD4 CAR constructs.

P045

Tetracycline Enabled Self-Silencing Adenovirus (TESSA™) – a versatile and high-yielding scalable platform for rAAV manufacture

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Recombinant adeno-associated virus (rAAV) is the vector of choice for *in vivo* gene therapy, however, efficient manufacture of rAAV to meet pre-clinical and clinical demands remain challenging. To solve this critical manufacturing challenge in generating high yield and high quality rAAV vectors, we developed a novel self-silencing helper adenoviral vector system entitled 'Tetracycline-Enabled Self-Silencing Adenovirus' (TESSA™) to deliver the AAV genes and adenoviral 'helper' functions, into HEK293 cells, for efficient rAAV manufacture without adenovirus contaminations. TESSA™ is a robust and versatile platform to produce rAAV, either by using two TESSA™ vectors to deliver AAV rep/cap and the rAAV transfer genome, or wherein the rAAV transfer genome is delivered via stable integration in the cell's chromosome, plasmid transfection, or co-infection with existing rAAVs for vector propagation. This multitude of approaches to rAAV manufacture using TESSA™ enables researcher to produce cost-effective rAAV vectors, both rapidly and scalable. The TESSA™ platform yields >30-fold more rAAV vectors compared to the triple plasmid transfection process across a wide range of AAV serotypes including AAV1-9 & rh10, and generating rAAV vectors at productivities of >1E+6 vector genome copies (GC) per cell. We successfully demonstrated that up-scale production of rAAV6 and rAAV2 in 50 L and 200 L bioreactors runs using TESSA™ is capable of yielding >7E+11 GC per mL of cell culture and >1E+17 GC of Drug Substances. Importantly, TESSA™ enables significant improvement in the efficiency of rAAV genome packaging with AUC analysis showing >60% of full capsids after affinity-capture and >95% of full capsids after ion-exchange chromatography. To ensure the economical manufacture of clinical rAAV gene therapy drugs and accelerated product approval, the TESSA™ technology is integrated with intricate in-house testing capabilities for assay development, biosafety, viral clearance, and product release testing.

P046

Enhancing EV-AAV incorporation: Insights into natural loading and exogenous binding strategies for improved gene therapy vector delivery

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Adeno-associated viruses (AAVs) are one of the leading gene therapy vectors for clinical applications. However, AAV serotypes are limited by their inherent tropisms to specific tissues, and the presence of neutralizing antibodies (NAbs) in a patient's bloodstream can prevent efficient biodistribution of AAVs and make repeated treatment challenging. It has been shown that a small proportion of the AAVs incorporate into extracellular vesicles (EVs) to evade immune detection. These EV-AAVs have several advantages over standard AAVs including protection from NAbs, improved transduction efficiency and ability to co-package additional molecules for improved targeting or immunomodulation. Nonetheless, EV-AAV candidacy for clinical therapy is limited by low levels of natural incorporation into vesicles. EVs are lipid nanoparticles produced by cells that can be subdivided into three groups based on origin: exosomes, microvesicles and apoptotic bodies. It is the goal of this study to characterize EVs that naturally incorporate AAVs, to inform future engineering strategies in increasing EV-AAV yield. Here, using a flow nanoanalyzer and two independent staining methods, we show that the percentage of EVs that incorporate AAVs is less than 2%, highlighting the need for the development of engineering strategies that can increase the incorporation of AAVs inside EVs. Evaluation of three different serotypes – serotypes 2, 8 and 9 – demonstrates similar (low) percentages of AAVs being secreted in association with EVs. Ultracentrifugation separation of large (20k pellet) and small (100k pellet) EVs, reveals that larger vesicles incorporate AAVs more efficiently. The biogenesis pathway of EV-AAVs was investigated using siRNA knockdown, as well as chemical inhibition of exosome and microvesicle release pathways. To explore the potential incorporation of AAVs into EVs via interactions with common EV markers, we conducted a study wherein 12 different markers were overexpressed in HEK293T producer cells. Our findings revealed that the overexpression of TSPAN2, CD63, and PTTG1IP significantly enhanced the yield of EV-AAVs. Pulldown experiments reveal that EV-AAVs are enriched in phosphatidylserine, but surprisingly devoid of tetraspanin markers. In order to overcome the challenging unknowns of endogenous loading, initially we evaluated exogenous binding of AAVs at the surface of EVs. Laminin Receptor and AAV Receptor (AAVR) were both evaluated for loading capabilities. AAVR engineered EVs were able to bind AAVs efficiently and showed protection from NAbs *in vitro*. These findings contribute valuable insights for future advancement of EV-AAV candidacy for clinical gene therapy applications.

P047

Leveraging a spectral cytometry immuno-surveillance assay for orthogonal detection of AAV-reactive donors in the general population and beyond

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Adeno-associated viral vector (AAV) gene therapy is a clinical reality, aiming to bring life-altering treatments to subjects afflicted by genetic diseases. However, natural exposure to wild-type AAV results in ~30-70% seropositivity for neutralizing antibodies (NAb), restricting enrollment of clinical trial participants. While NAb assays are extremely useful, studies indicate they may not fully capture AAV immunity status and can fail to identify nuances in cross-reactivity to other serotypes. Traditional immunological assays detecting NAb titers or reactive T-cell frequencies by ELISpot are core assessments of participants in ongoing AAV clinical trials, so we aimed to develop a complementary immunophenotyping assay harnessing the high-dimensional power of spectral cytometry. This 40+ color panel works in tandem with *ex vivo* whole blood AAV stimulation to detect upregulation of activation-inducible markers across a variety of immune cell types; adaptive T and B cells, antigen-presenting cells that drive these adaptive responses, and innate subsets that purportedly sense AAV capsids. We then leveraged this deep-phenotyping assay to identify naturally occurring AAV8-reactive donors among a cohort of healthy donors via costimulatory molecule upregulation on immune cell subsets following incubation with whole AAV8 capsid. AAV8 adaptive immunity was confirmed by classical readouts of neutralizing and binding antibodies, and capsid-specific ELISpot. Additionally, we were able to visualize increased uptake of AAV8 capsid into innate cells from reactive versus non-reactive donors via imaging cytometry. Overall, this assay demonstrates the ability of modern, high-dimensional immunological approaches to recapitulate clinical ones – furthering our understanding of the breadth of natural immunity to AAV.

P049

Silence and replace as potential treatment for Alzheimer Disease: in concert lowering toxic APOE and augmenting protective APOE

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Alzheimer Disease (AD) is the prevailing type of dementia affecting individuals aged 65 and above (~50 million), characterized by progressive deterioration of memory and cognitive function. The primary genetic risk factor of late-onset AD (LOAD) is the Apolipoprotein E4 (APOE4) variant. APOE is expressed in the liver and brain, facilitating lipid transport and homeostasis. Among the general population, the *APOE* gene exists as three polymorphic alleles, with APOE3 as the predominant variant, followed by APOE4 and APOE2 (79>13.3>7.3%). APOE4 is present in

45-60% of all AD cases and is associated with increased risk and earlier disease onset, whereas APOE2 is considered neuroprotective. Previous studies demonstrated that depleting APOE4 in humanized (h) APOE-AD mouse models can prevent neurodegeneration. We aim to develop an AAV gene therapy approach to treat LOAD patients by simultaneously silencing toxic APOE4 and overexpressing a protective APOE-variant. Previously, we conducted an *in vitro* screen to identify the best APOE silencing microRNA candidates (miAPOEs), as well as to identify ideal protective APOE-variants. A potent reduction of hAPOE4 mRNA and protein was observed in brains of *hAPOE4*-transgenic mice dosed intrastriatally with AAV-miAPOEs.

The best protective APOE-variants were examined *in vivo* by intrastriatal AAV delivery in a human Tau overexpressing transgenic model; P301S Tau-transgenic mouse. Seven months post-injection, elevated expression of the protective APOE-variants were observed in the brain, resulting in a significant decrease of pTau181 in the hippocampus.

Finally, the most potent miAPOEs and protective APOE-variants were selected and implemented into a combined construct to function in concert. The combined constructs that simultaneously encode miAPOEs and protective APOE-variant transcripts were validated in an *in vitro* screen to demonstrate miAPOE efficacy as well as APOE-variant expression. A selection of these constructs were tested *in vivo* via intrastriatal delivery of AAV vectors in wild-type mice. One month post-injection, transgene expression levels derived from AAV vectors encoding combined constructs or only miAPOEs or a protective APOE-variant were compared. The results showed that mice injected with AAV vectors encoding the combined constructs or only miAPOE displayed similar elevated levels of miAPOE in their brains. However, APOE mRNA and protein levels observed in the brains of mice injected with AAV vectors encoding combined constructs were superior to the mice injected with AAV vectors only encoding a protective APOE-variant.

In conclusion, simultaneous silencing of toxic APOE and overexpressing a protective APOE-variant is a promising gene therapy approach for AD.

P050

Neurohepatic promoters, a novel path towards AAV-mediated mitochondrial diseases therapy

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Inherited mitochondrial diseases are a group of rare genetic disorders that affect the energy-producing organelles in the cells. These disorders are characterized by a dramatic reduction of the adenosine triphosphate (ATP) production, causing a range of symptoms and health problems. The most affected organs by inherited mitochondrial diseases are the ones that have high energy requirements, like the liver and the central nervous system (CNS). These group of disorders have no cure and current treatments are focused on managing symptoms and supporting overall health. This can include medication, nutritional support, physical therapy, and other medical interventions. Currently, there is no cure for inherited mitochondrial diseases, but ongoing research is focused on developing new treatments and improving patient outcomes.

Gene therapies using recombinant adeno-associated virus (rAAVs) have revolutionized the treatment of some monogenic diseases. However, their application to multisystem indications, like

inherited mitochondrial diseases, requires optimization. On one hand, the use of vectors with ubiquitous promoters administered systemically can trigger immune responses that cause significant adverse effects. On the other hand, using vectors with specific promoters for each affected tissue would require the development of independent rAAVs, significantly increasing their development costs and safety profile. Therefore, this project proposes the development of rAAVs carrying a hybrid promoter specific to the target tissues that could overcome these drawbacks.

To achieve this, we first tested different constructs combining a neuro-specific and a hepato-specific promoter driving the expression of a reporter transgene in hepatic and neuronal cell lines. We selected the two most efficient combinations for *in vivo* testing in wild-type mice by local or systemic administration, using AAV9 or AAV9P31 as delivery vehicles encoding the mCherry reporter transgene. Animals were sacrificed 21 days after vector injection and vector biodistribution and transgene expression were analysed in different organs. Moreover, mCherry expression was analysed by immunofluorescence and immunohistochemistry together with cell specific markers.

The results indicate that our hybrid promoter represent a viable alternative for selective expression of the transgene showing robust expression in the two target tissues in one of the two tested combinations. Interestingly, mCherry expression was stronger in the CNS with the hybrid promoter compared to a neuro-specific promoter alone.

In summary, design of hybrid promoters specific to more than one target tissue has potential application to multisystem diseases with hepato-neurological involvement, including inherited mitochondrial diseases.

P051

Duration and specificity of gene transduction following intravenous delivery of AAV9-cBIN1 in minipigs

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Heart failure (HF) is a highly morbid disease with limited treatment options. Despite HF being a disease of failing muscle, no current HF therapeutic was primarily designed to target failing heart muscle itself. In individual failing cardiomyocytes, HF is associated with disrupted transverse-tubule microdomains, limiting organization of the calcium handling machinery and cardiac function. Microdomain disruption is caused by reduced transcription of a membrane scaffolding protein cardiac bridging integrator 1 (cBIN1), weakening beat-to-beat calcium transients and impairing cardiac function. Fortunately, impaired microdomains can be restored by cBIN1 replacement therapy introduced by adeno-associated virus 9 (AAV9) mediated gene therapy to rescue heart failure. In a minipig model of non-ischemic dilated cardiomyopathy and heart failure, we found low dose intravenous delivery of cBIN1 gene therapy is efficacious for at least 6 months. However, it is not known how long the exogenous cBIN1 transgene persists in minipig hearts, as well as its expression in other organs particularly liver and skeletal muscle. In this study, we explored the transduction duration of AAV9-cBIN1 in minipig hearts and other organs. 8-10 months old Yucatan minipigs with tachycardia-induced heart failure received an intravenous dose of PBS or AAV9-CMV-cBIN1-V5 ($6x10^{11}$ vg/kg). Weekly blood work was obtained to evaluate liver and renal function. Animals were terminated 6 months after injection and tissue samples were obtained from heart and other organs for histopathology and gene expression analysis. Realtime PCR was used to quantify exogenous mouse cBIN1-V5 gene using a

probe detecting V5 and endogenous cBIN1 using a probe specific for porcine cBIN1 gene. Our results indicate that throughout the 6-month period, no organ dysfunction was noted by blood test, or with histopathology at 6 months. At 6 months, ddCt results of *V5/HPRT1* indicate that AAV9 effectively transduces exogenous cBIN1-V5 (24.1 ± 5.4 fold above PBS controls) in hearts without detectible transduction elsewhere. dCt results of *V5/cBIN1* (porcine) indicate that exogenous cBIN1-V5 expresses at 20.9 ± 1.8 % of endogenous cBIN1 gene in minipig hearts which is the level of cBIN1 overexpression needed to restore cardiac function. In conclusion, we identified that a single low dose intravenous therapy of AAV9-cBIN1 can reach efficacious levels for at least 6 months in minipig hearts without off target transduction in other organs.

P052

Tailoring AAV vectors for gene therapy of inner ear disorders by directed evolution

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Hearing loss (HL) affects approximately 20% of the global population and the treatments are currently limited to hearing aids and cochlea implants. Gene therapy offers a possibility to prevent or even cure HL. With the aim to optimize the adeno-associated virus (AAV) vector system for inner ear directed gene therapy, we generated AAV peptide display libraries based on the AAV1, AAV2 and AAV6 capsid backbones. The libraries present random unique 7-mer peptide inserts at variable region VIII of the capsid protein with high diversities ranging from 80,000-622,000 (maximum likelihood estimate, MLE). We conducted high-throughput *in vivo* selection screens of these libraries in the inner ear of adult mice, testing alternative administration routes that demand overcoming robust biological barriers.

The target tissue is the organ of corti – located within the cochlea – and comprises the crucial mechanosensory hair cells (HCs) of the inner ear, the supporting cells (SCs) and the underlying spiral ganglion neurones (SGNs) which are commonly affected in HL. Interestingly, while distinct variants were found to be accumulated to up to 5% for AAV2-based variants or up to 2.5% for AAV1-derived capsids, we did not observe any enrichment for specific variants from the AAV6 peptide display library after two rounds of *in vivo* selection. Top candidates were produced as vectors and show a range of expression patterns in the adult mouse cochlea. Infection of both inner and outer hair cells as well as SGNs was seen. Several of the variants appeared to transduce only inner hair cells. A range of different expression patterns were seen in the cochlear lateral wall including expression in whole stria vascularis and more limited expression in the vasculature of the cochlea. In addition, different intensities of fluorescent transgene expression suggest differential efficacy in delivery or vector uncoating within the inner ear.

Thus, we report on a set of promising new variants with distinct features developed by *in vivo* high throughput selection screens for improving inner ear directed gene therapy.

P053

Development of novel parvovirus-derived vectors for gene therapy

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Carbon Biosciences is developing a catalog of novel therapeutic viral vectors derived from autonomous parvovirus species. While AAV vectors derived from dependoviruses are the basis of approved gene therapy products, there has been a paucity of R&D efforts around other parvovirus genera containing different biological properties. In addition to the dependoparvovirus genus, the large parvovirus family provides a deep reservoir of potential vectors for human gene therapy, expanding the genetic diversity beyond AAV. Although many autonomous parvovirus species do not naturally infect humans, the capsids remain capable of transducing human cells. Therefore, members of this virus family may be utilized as therapeutic vectors that bypass the pre-existing immunity and cross-reacting antibodies against viruses that natively infect humans, thus increasing the eligible patient population for gene therapies. Furthermore, whereas AAV capsids are limited to approximately 4.7 Kb genomes, the virion genomes of autonomous parvovirus are generally larger and the capsid may accommodate vector DNA exceeding 6 Kb. This increased genetic capacity extends the range of treatable diseases that requires open-reading frames that AAV cannot accommodate and also enables new gene editing modalities. Additionally, larger, cis-acting elements e.g., tissue-specific promoters or homology arms, can be included in the vector genome. Here we provide evidence of successful trans-packaging of a DNA vector genome containing AAV2 ITRs into two novel autonomous parvovirus capsids. The manufacturability of the proposed hybrid parvoviral vector, based on DNase-resistant particles, is similar to those obtained with different AAV serotypes. Furthermore, these hybrid parvoviral-derived vectors show robust transduction of different human cell systems, highlighting the enormous potential of autonomous parvovirus-derived vectors for gene therapy applications. Development of novel parvovirus vectors may offer differentiated tropism, immunological advantages, and optimized transgenes while simultaneously leveraging the de-risked safety profile, manufacturing ecosystem, regulatory advantages, and lessons learned from decades of AAV research.

P054

Characterisation of the inflammatory profile of AAV6 in a pig and human model

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Adverse effects related to inflammatory reactions compromise the safety and long-term efficacy of retinal gene therapy. We examined retinal immune cell activation following subretinal injection of AAV6 and AAV8 in Yucatan minipigs and compared it with the effects of these vectors on microglial culture. An overt inflammatory response was found in 2 of 3 pigs injected with a moderate to high dose of AAV6-CAG.EGFP (4e11 total VG per eye) while pigs injected with AAV8-EGFP or PBS did not show clinical effects. Invading phagocytic mononuclear cells, cytokine levels in the ocular fluid, as well as anti-transgene and anti-capsid levels in PBMCs and splenocytes were measured to help characterise the nature of the reaction. In parallel cytokine secretion following AAV6 and AAV8 administration in vitro – using induced microglial-like cells derived from human iPSCs and the immortalized microglial HMC3 cell line - were evaluated. The results highlight the risk for retinal inflammation and the need to further study the serotype-specific response of microglial cells in rAAV gene therapy studies.

P055

Small non-coding RNAs to overcome cell restriction factors and improve AAV vector transduction

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AAV vectors are now widely used as a gene transfer tool in basic research and in translational gene therapy. However, recent clinical trials highlighted the limits of AAV transduction efficiency, which impose high dosage treatments and the consequent exposure to severe vector related toxicities. The gaps in our understanding of the biology of these vectors and of the cell restriction factors that constrain their efficacy, needs to be urgently filled.

To systematically identify the host cell factors involved in virus fate after infection, we performed a high-throughput screening using a genome-wide siRNA library identifying components of the double-stranded DNA break repair as regulators of rAAV genome processing. More recently, we performed a high-throughput screening using a genome-wide library of human microRNA mimics (988 mature sequences, miRBase 13 - Dharmacon) in AAV2-Luciferase transduced HeLa cells. Using this approach, 51 microRNA mimics were shown to increase AAV transduction by more than 4-fold (up to 23-fold change) while 26 microRNA mimics significantly decreased AAV transduction. Hsa-miR-329 and hsa-miR-362-3p, which share the same seed-sequence, were identified as the most effective microRNAs at increasing AAV transduction in HeLa cells. Transcriptomic analysis on total RNA extracted from HeLa cells transfected with 3 top hit

microRNAs identified several hundred transcripts significantly downregulated. The direct comparison of these results with those obtained from the screening of siRNAs for AAV2-mediated transduction, highlighted the involvement of at least three categories of proteins able to interfere with AAV transduction, including i) proteins involved in sensing of DNA damage, induction of DDR and reactivation of cell cycle checkpoints, such as MRN proteins Mre11, Rad50 and Nbs1, and MDC1; ii) proteins of the endocytic pathway, of which arms of clathrin mediated endocytosis pathway appear to exert an inhibitory effect on transduction; iii) molecules that regulate the epigenome, the prototype of these being SETD8, target of a top hit of siRNA screening. All together, these findings revealed the power of exploiting ncRNAs to understand the molecular bases of AAV permissivity and to modulate transduction. Of note, the most effective siRNAs and miRNAs are independent from the virus serotype and cell types and are equally effective for single-stranded and self-complementary AAV vectors, offering a potent tool to improve AAV-mediated gene transfer.

P056

Optimized display of bulky peptides in Adeno-associated virus

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Capsids of the Adeno-associated virus (AAV) provide an excellent basis for the display of proteinaceous targeting entities, with the aim to deliver transgenes to cell types that are refractory to the unmodified wild-type virus. Previously, we and others have harnessed this technology for the display of short peptides and exemplified its great potential to alter AAV tropism in vitro and in vivo. Here, we report complications that we encountered with specific peptides in various AAV backbones and describe accompanying solutions on how to rescue production of the engineered particles and unlock their full potential. This includes our discovery of an enhanced sensitivity of capsids displaying arginine-containing peptides to digestion by even trace amounts of trypsin in cell media, which can be counteracted by switching to trypsin-free production in suspension cells. Secondly, display of cysteine-containing peptides such as the RGD-binding peptide RGD4C (CDCRGDCFC) can trigger formation of inter-particle disulfide bonds and capsid aggregation during standard virus purification process, which can be resolved by adding reducing reagent such as dithiothreitol. Finally, we found that insertion of some already known functional peptides, like the glioblastoma-targeting peptide linTT1 (AKRGARSTA) or the blood-brain-barrier-penetrating peptide Angiopep-2 (TFFYGGSRGKRNNFKTEEY), destabilized the AAV capsid. Importantly, the generation and efficiency of viruses displaying such peptides can be boosted by creating mosaic capsids in which the engineered capsid protein is diluted by wild-type counterparts. Collectively, we anticipate that our observations and solutions are pertinent for other targeting sequences as well and will thus widely foster the production and use of peptide-modified AAV vectors.

P057

Achieving successful therapeutic levels of FVIII at low vector doses via enhancing AAV capsid design

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Adeno-associated virus (AAV)-mediated gene therapy has emerged as a promising approach for treating hemophilia diseases, but pre-existing anti-AAV neutralizing antibodies (NAbs) pose challenges. AAV5 exhibits minimal antigenicity with the lowest prevalence of pre-existing NAbs in the population, however, its infectivity is relatively poor, requiring high vector doses (6E13 vg/kg) as recently reported by clinical investigators [1,2].

In this study, we aimed to improve AAV5's hepatocellular infectivity through VP1/VP2-N-terminal swapping and VP3 modification. Swapped AAV5 vectors, generated by replacing VP1/VP2-N terminals with those from AAV2, AAV7, AAV8, and AAV9, demonstrated 4.5-9.6-fold increased transgene expression *in vitro*, however, no significant differences were observed in liver after systemic injection. To further enhance liver tropism, we inserted the additional VR-I sequences from AAV6 and AAV8 into AAV5 VP3 at position of VR-VIII, resulting in the generation of AAV596 and AAV598. Upon tail vein injection into C57BL/6 mice, AAV596 and AAV598 vectors exhibited specific liver targeting, leading to a substantial 13.4-fold and 7.5-fold increase in LacZ gene expression, respectively, with lower expression observed in other organs. Subsequently, AAV596 and AAV598 were utilized for the treatment of hemophilia A mice, resulting in a significant up to 38.9-fold increase in Factor VIII (FVIII) expression (4E12 vg/kg) in blood plasma compared to the original AAV5 vector, while retaining low antigenicity of AAV5.

Thus, the chimeric AAV596 and AAV598 vectors, which demonstrate specific liver targeting and possess low antigenicity, hold great potential as ideal candidates for gene therapy in hemophilia diseases and other genetic liver disorders.

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P058

Developing a multi-functional and multi-lineage human platform based on 3D engineered muscles to assess neuromuscular gene therapies

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Skeletal muscle architecture and function is impaired in several neuromuscular and musculoskeletal disorders, including muscular dystrophies: severe, incurable inherited disorders characterised by muscle wasting, limited mobility and premature death. Although adeno-associated virus (AAV) mediated gene therapy is emerging as a promising therapeutic strategy for muscular dystrophies, clinical success remains limited, due to modest level of efficacy or adverse events post treatment (often not observed in pre-clinical animal studies). These shortcomings can be mitigated by robust, human(ised) models predicting cell-/tissue-specificity, toxicity and efficacy during early AAV gene therapy development. To this aim, we describe here the development of an *in vitro* human platform based upon 3D engineered skeletal muscles to assess gene therapy vectors with high efficacy, low toxicity and selective tropism. We first assessed if our 3D, human induced pluripotent stem cell-based platform to engineer skeletal muscle constructs would be compatible with direct in vitro AAV transduction and then explored dose-dependent transduction efficiency and transgene expression pattern of several natural AAV serotypes. Morphological and molecular assessments of GFP reporter transgene expression demonstrated dose-dependent signal increase in transduced engineered 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 four 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 bi-lineage 3D muscles containing isogenic myofibers and motor neurons. Analyses of transduced constructs indicate preferential tropism of neuronal-specific rAAVs in SMI32-positive motorneurons compared to myosin-positive myotubes, validating fidelity of our platform vs. conventional assays in rodents. Additionally, we also confirmed that neuronal-specific rAAVs outperformed natural serotypes such as AAV8 by in transducing motorneurons in human 3D cultures. Finally, we will present current efforts to go beyond multi-lineage, setting up a multi organ(oid) platform to perform similar assays in tissues known to be off targeted by AAVs, as well as strategies to introduce therapeutically relevant transgenes to be tested in this innovative, *quasi-vivo* platform.

P059

Bioconjugated adeno-associated virus-derived vectors: an alternative to enhance gene expression in targeted tissues.

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Adeno-associated virus (AAV) gene therapy represents decades of biological and clinical research and it has become clear that challenges need to be overcome to exploit all the potential of these vectors. Here, we have a developed chemical bioconjugation strategy to selectively modify lysine (K) or tyrosine (Y) residues on recombinant AAV capsids with carbohydrate ligands (BioAAV). The covalent coupling reaction occurs by nucleophilic addition of an amino group of lysine with isothiocyanate ligands or by aromatic electrophilic substitution of the phenol of tyrosine residues with diazonium salt ligands. Several ligands such as *N*-acetylgalactosamine (GalNAc) and mannose were used and the bioconjugated AAV vectors were evaluated for different targets such as the liver, the retina, and the brain.

After validation of the chemical coupling using a panel of analytical assays, we evaluated *in vivo* - in rodents and non-human primates - the efficiency of these BioAAV2 (all vectors carrying an Enhanced green fluorescent protein (eGFP) reporter gene expression cassette under the control of a ubiquitous promoter).

Using our newly developed bioconjugated rAAV2 vectors we observed *in vivo* - after systemic injection in the liver, subretinal injection in the retina and intrastriatal injection in the brain - a significant impact on vector transduction efficiency and expression of the gene of interest. In all targeted organs, an increase in transduction area and in the number of transduced cells was observed for BioAAV2 *versus* unmodified rAAV2.

Thus, we believe our results reveal that bioconjugation of lysine or tyrosine onto therapeutic vectors is a promising alternative to genetic engineering methods for enhancing organ tropism and protein expression in specific cells. This strategy may pave the way for the use of these chemically modified vectors for gene therapy treatments targeting liver diseases, glaucoma, optic neuropathies and neurodegenerative diseases.

P060

A micro-RNA regulated AAV vector prevents the cardiotoxicity induced by transgene overexpression following FKRP gene transfer

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The deficiency in Fukutin-Related Protein (FKRP) is the cause of diseases ranging from the very severe Walker-Warburg syndrome to the milder and more frequent Limb Girdle Muscular Dystrophy (LGMD) R9. FKRP, in the Golgi apparatus, is one of the proteins acting in the complex multi-step process of alpha-dystroglycan (aDG) glycosylation. Its precise role is the adding of ribitol-phosphate to the elongating glycosylation chain. The integrity of aDG glycosylated chains is crucial in the anchoring of muscle fibers in the extracellular matrix. The disruption of the proper anchoring decreases the resistance of fibers to the stress induced by muscle contractions.

AAV-mediated FKRP gene transfer was previously shown to be efficient in reducing muscle dystrophy in animal models of FKRP deficiency. However, overexpression of FKRP in the heart of wild-type rats following administration of AAV9-FKRP induced cardiac damages, eventually leading to animal death. We characterized the mechanisms involved in the cardiotoxicity induced by AAV9-FKRP and found out that an important amount of FKRP protein was expressed and accumulated in the transduced cardiac tissue, leading to activation of endoplasmic reticulum stress, decrease of autophagy, and activation of different pathways of cell death. To overcome such cardiotoxicity in FKRP gene transfer studies, we designed an AAV vector harboring in 3' position of the FKRP coding sequence the target sequence of a cardiac-specific micro-RNA, miR-208a. The use of this vector was shown to be safe, avoiding any cardiac damage. These observations highlight the importance of controlling transgene expression in the heart in gene therapy studies.

P061

A novel platform of chemically modified 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, these approaches also have a number of shortcomings. First, genetic modification often reduces yield and potency of AAV vectors. Second, since only small peptides can be accommodated into the capsid structure, modified variants may have only weak or promiscuous binding to their cellular targets. Third, the cellular receptor(s) of genetically modified AAVs are generally not known a priori, making translatability across species difficult.

Here we describe an alternative approach to capsid engineering based upon modification of the AAV capsid through 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.

To illustrate the flexibility of our approach, we describe how, depending on the ligand we choose, 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. Furthermore, we provide functional evidence of the in vivo efficacy of our targeted vectors using an experimental pain model.

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.

P062

Nanobodies inserted into different surface loops retarget AAV5 and AAV9 to specific cells

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The broad tissue tropism of most AAV vectors is often addressed by driving transgene expression by a tissue- or cell specific promotor. However, this often requires the use of high doses and may be accompanied by undesired side effects. We address these limitations with a direct targeting approach using membrane-protein-specific nanobodies. These single antigen-binding domains are derived from camelid heavy-chain antibodies and are characterized by small size and high solubility (1). Through genetic engineering, we inserted nanobodies specific for different membrane proteins into the GH2/3 or GH12/13 loop of the VP1 capsid protein of AAV5 and AAV9. The display of a nanobody in either loop of the AAV capsid significantly enhances the transduction of HEK cells that express the corresponding membrane protein, e.g., PD-L1, CD38, P2X7, and CD73. We also demonstrate the feasibility of simultaneously inserting two different nanobodies (αCD38 and αPD-L1) into the GH2/3 and GH12/13 loop of the same VP1 capsid protein. As a result, the modified AAV displaying bispecific nanobodies exhibits enhanced transduction efficiency of cells expressing either CD38 or PD-L1. These findings illustrate the versatility of nanobodies as effective and broadly available targeting domains to improve AAV gene therapy.

P063

Engineering of myotropic AAV vectors for expression of broadly neutralizing anti-HIV-1 antibodies

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Since the recognition of the human immunodeficiency virus (HIV) as the causative agent of AIDS and the associated pandemic in the early 1980´s, ~85 million people have become infected with the virus. Most infections are efficiently prevented and controlled by our immune system even though they might become chronic. Yet, in immunocompromised individuals, latent and otherwise controlled viruses often resurge as acute infections that can cause severe symptoms and ultimately fatality.

To date, conventional vaccination-strategy interventions failed, probably due to the fact, that the by the virus exposed proteins (gp120/gp41) are highly glycosylated and the high error rate of the viral reverse transcriptase, resulting in escape mutations. Nevertheless, in a small proportion of HIV-1-infected individuals, a persistent immune-mediated control of viremia occurs early after infection (so-called elite controllers) or after discontinuation of an anti-retroviral therapy (post-treatment controllers).

These findings lay a pivotal foundation for experimental, clinically relevant strategies to deliberately boost the induction of HIV-1 control in humans even prior to infection. Particulary promising is “vectored immunoprophylaxis” (VIP), in which broadly neutralizing anti-HIV antibodies (bNAbs) are encoded in and expressed from viral gene delivery vectors, such as Adeno-associated virus (AAV). However, despite the great promise of this approach and numerous encouraging in vivo data, clinical success will depend on the comprehensive optimization of the AAV vector system for bnAb expression.

To this end, we harness our latest generation of muscle-specific (myotropic) AAV capsid variants, which mediate highly specific and concurrently robust transgene expression in the entire musculature in various small or large animal species following peripheral delivery. This, in turn, promises a dual benefit for use in a VIP setting, namely, the ability to 1) repurpose the largest organ in the human body (skeletal muscle) into a bnAb prodution and secretion factory, and 2) concomitantly detarget the liver, which is the major off-target in systemic AAV gene therapies and has recently been critically involved in human fatalities during gene therapy trials.

To further enhance the efficiency, specficity and safety of our AAV-VIP platform, we are evaluating different AAV vector scaffolds, i.e., single-stranded (ss) or self-complementary (sc). While ssAAVs offer a greater capacity for foreign cargos of up to 4.8 kb, allowing co-expression of the heavy and light antibody chains from a single template, scAAVs provide faster kinetics and more robust expression. Concurrent optimizations aim at improving the bnAb expression-cassette and include the use of muscle-specific promoters or post-transcriptional regulatory elements. Finally, we assess permutations of our myotropic capsids including variants that allow minor expression in the liver, in order to study whether hepatic expression will induce a beneficial tolerance against the bnAb.

In our presentation, we will disclose the first results of these evaluations and comparisons, and offer informed guidelines for the design of future AAV-VIP vectors for prevention of HIV-1 and other viruses.

P064

Improvement of liver-tropic AAV capsids: neutralizing antibody-evading variants of sL65 allow re-treatment through serotype-switch while maintaining cross-species transduction

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Antibodies against AAV are a significant cause of patient exclusion in gene therapy trials and prevent redosing of treated patients. We previously reported the generation of the novel human liver tropic capsid sL65, isolated in humanized mice and validated in non-human primates (NHPs). The sL65 capsid shares a limited number of epitopes with capsids commonly used in clinical trials. However, its variable region I (VRI) is homologous to AAV2, AAV3B and LK03, making sL65 vulnerable to certain antibodies directed against these serotypes.

To enhance antibody evasion, we created ~15,000 variants of sL65 through VRI region mutations and screened them in humanized mice with and without neutralization by human immunoglobulins (IVIG). Remarkably, we identified candidates that evaded pre-existing neutralizing antibodies while maintaining potency in human hepatocytes. These engineered capsids also exhibited enhanced transduction efficiency in various human cell lines, including Huh7 and RC32 Hela cells. Interestingly, the capsid variants showed increased transduction efficiency in mouse hepatocytes, indicating potential cross-species translatability.

In order to explore redosing by serotype switching, we utilized serum from NHPs previously dosed with different liver tropic capsids, including AAV8, DJ, LK03, and sL65. As hypothesized, we observed that the sL65 variants were not neutralized in vitro by the sera of NHPs dosed with LK03. The in vivo confirmation of AAV redosing in NHPs will be presented in the upcoming meeting.

In summary, the targeted engineering of sL65 VRI led to decreased capsid neutralization by pre-existing neutralizing antibodies, offering the potential to treat a broader patient population and/or to re-retreat patients who previously received gene therapy with an unrelated serotype.

P065

Phage-guided CRISPR/Cas9 deletion of therapeutic gene targets in medulloblastoma: a novel tumour-targeted gene therapy approach

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Medulloblastoma are extremely heterogeneous solid malignant tumours that occur in the cerebellum or fourth ventricle of the brain. They are the most common paediatric brain cancer but, although cases do occur, are relatively rare in adults. Tumours are categorised into four main clinically distinct molecular subgroups: WNT, SHH, Group 3 and Group 4, with each having a key driver pathway of oncogenesis or specific genetic characteristics. Current conventional therapies include invasive neurosurgery, craniospinal radiotherapy and high-dose cytotoxic chemotherapy. Whilst this aggressive multi-modal treatment regime has improved the overall survival rate, this has remained unchanged for the last 30 years, varies drastically between subgroups and does not necessarily correlate with an improvement in the quality of life of survivors. Survivors have an unacceptably high risk of suffering both transient and permanent side effects from treatment including post-operative complications, neurological, cognitive, and intellectual deficits, endocrine disorders, infertility, and secondary cancers. As such, an alternative treatment that is safe, non-invasive, targetable, and efficient is desperately needed to improve survival rates and save the quality of life of young survivors. Whilst potential therapeutic targets have been identified in medulloblastoma, there have been issues with drug design, drug resistance and systemic side effects throughout the body of any potential therapeutics. Tumour-specific nucleic acid delivery, or gene therapy, offers a possible solution to many of these issues and would offer major advancement in the treatment of medulloblastoma. However, progress has been hindered by 1) a lack of tumour-selective delivery vectors capable of clinical systemic delivery routes, 2) the blood-brain barrier (BBB) and 3) issues with repeated administrations which are essential in achieving and sustaining a therapeutic response. Our group have developed a hybrid bacteriophage (phage)-based delivery system termed Transmorphic Phage/AAV (TPA), consisting of M13-derived phage capsid proteins encapsulating a recombinant adeno-associated virus 2 (rAAV2) genome expressing a transgene cassette and can specifically and efficiently target only tumour cells while avoiding healthy ones. This is facilitated by ligand-directed targeting using the double cyclic RGD4C motif that is displayed on the phage capsid which binds to αvβ3 and αvβ5 integrins which are overexpressed on tumour cells and tumour vasculature, but minimally expressed on healthy cells. Importantly, this particle has the ability to cross the BBB after systemic administration. Here we show that the large size CRISPR/Cas9 gene-editing system can be cloned into the rAAV2 transgene cassette of the TPA vector to generate an efficient novel gene therapy tool against medulloblastoma. We have produced multiple particles with the CRISPR/Cas9 system targeted to various different therapeutic gene targets within medulloblastoma and have shown successful TPA-mediated gene delivery and subsequent gene knockdown *in vitro*. Importantly, we report significant targeted anti-tumour responses *in vitro* after treatment with the tumour targeted particles. Our novel CRISPR/Cas9 TPA delivery system presents a promising tumour-targeted medulloblastoma therapy that facilitates systemic clinical administration with limited expected side effects.

P067

Preclinical efficacy & safety of AAVrh.74-PKP2a (RP-A601): gene therapy for PKP2-associated arrhythmogenic cardiomyopathy

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Pathogenic loss-of-function variants of the *PKP2* gene, which encodes for the desmosomal protein Plakophilin 2 (PKP2), constitute the largest proportion of known genetic causes of arrhythmogenic cardiomyopathy (ACM or PKP2-ACM) a progressive, inherited, autosomal dominant disorder. PKP2-ACM manifests most commonly in young adults as extensive cardiomyocyte loss, myocardial fibrofatty infiltration, frequent ventricular arrhythmias, systolic dysfunction, and significant risk of sudden cardiac death (SCD). Current therapeutic options include pharmacologic agents to reduce arrhythmia risk, cardiac ablation, and implantable cardioverter-defibrillators (ICD), none of which alter disease pathophysiology or progression. For late-stage disease, cardiac transplantation is often warranted. To address the substantial unmet need, RP-A601 (AAVrh.74-PKP2a), a recombinant adeno-associated viral vector containing the coding sequence of human plakophilin-2a, is under clinical development as a potential therapeutic for PKP2-ACM patients.

Intravenous AAVrh.74-PKP2a was evaluated in a well-characterized cardiomyocyte-specific PKP2 conditional knockout mouse model of ACM (PKP2-cKO). Robust efficacy in mitigating the ACM disease phenotype was observed across a range of doses and timepoints. A series of studies demonstrated that AAVrh.74-PKP2a at a dose of 6×10^{13} vg/kg resulted in robust PKP2 expression, 100% survival up to 5 months (longest time point evaluated; vs 100% mortality by day 50 in PKP2-cKO controls), stabilized right ventricular area, mitigation of fibrosis, robust benefit across multiple measures of cardiac function (including right ventricular velocity time integral, ejection fraction, fractional shortening) and substantial reduction in isoproterenol-induced arrhythmia burden. Importantly, robust long-term cardiac effects associated with hPKP2a expression were observed even when AAVrh.74-PKP2a was administered after disease onset.

Subsequent IND-enabling mouse and nonhuman primate safety and toxicology studies revealed appropriate vector DNA biodistribution consistent with previously published findings. Transgene mRNA was 10-100-fold enriched in the heart relative to all other organs, including liver. Clinical pathology and histopathology analyses revealed that AAVrh.74-PKP2a was well tolerated and safe across multiple studies up to (and including) doses of 3×10^{14} vg/kg.

Collectively, the preclinical efficacy and safety package strongly support the clinical development of RP-A601 as a potential therapeutic in patients with PKP2-ACM. The IND package was endorsed by the FDA in May 2023 and activity supporting initiation of a first-in-human clinical trial is ongoing.

P068

Development of cardiotropic AAV vectors by *in vitro* selections of random peptide libraries

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Adeno-associated viral (AAV) vectors are suitable to deliver genes into tissues, hence making them a powerful tool in gene therapy, and are already clinically applied for various diseases. Targeting of specific cells or organs can be achieved by exploiting the natural occurring tropism of different serotypes and modifying the viral capsid. Until now, AAV serotype 9 (AAV9) and AAV6 exhibit the highest transduction rates of murine cardiomyocytes *in vivo* and neonatal rat ventricular cardiomyocytes (NRVCMs) *in vitro*, respectively. It is desirable to generate AAV variants which work in both *in vitro* and *in vivo* models to facilitate translational research. In this study, we developed novel cardiotropic AAV vectors by selecting capsid libraries with random heptapeptides displayed on the capsid surface on NRVCMs. We systematically screened AAV variants derived from selections on both the genome and transcript level for their cell transduction and transgene expression efficiency. By that, we detected promising variants based on the AAV6 or AAV9 capsids with up to 7- and 1.6-fold higher *in vitro* transgene expression ability, respectively, compared to the AAV6 wild type capsid. Interestingly, AAV9 variants with sufficient transduction and transgene expression could be detected, which the AAV9 wild type was not capable of. Our results present an expansion of the AAV vector toolbox and demonstrate the improvement of AAV-mediated *in vitro* gene delivery into NRVCMs by capsid modification. We consequently anticipate our vectors to facilitate cardiac research *in vitro* and, finally, they might serve as cardiotropic AAV variants for *in vivo* applications.

P069

Characterization of the role of the transcriptional regulator Senataxin on AAV-mediated transgene expression

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Senataxin (SETX) is a highly conserved helicase involved in a large variety of biological processes, such as transcriptional regulation, pre-mRNA processing and maintenance of genomic integrity. Recently, loss-of-function in vitro screening experiments identified SETX as a factor that negatively regulates infection by HBV and other viral systems with episomal DNA. Preliminary data indicated that this restriction takes place at the level of transcription. In this study, we have investigated the role of SETX on rAAV transgene expression in vitro and in vivo.
For the in vitro studies, SETX expression was first downregulated in HepG2 cells with a shRNA and then cells were transduced with either single stranded (ss) or self-complementary (sc) rAAV3B vectors encoding mCherry reporter transgene. SETX downregulation significantly increased transgene expression after transduction with both ss and sc vectors, indicating first that SETX

interfered with AAV-mediated transgene expression and that its inhibitory activity took place after the synthesis of the double stranded genome.
In vivo, we compared SETX expression in the liver and brain of WT mice to that of control mice dosed with saline after systemic administration of an AAV-GFP. Protein expression by immunohistochemistry showed an increase of SETX expression in both tissues upon AAV transduction compared to control mice. SETX expression was mainly nuclear but cytoplasmic localization was also detected in both tissues. In a different study, transient silencing of SETX expression by siRNA was evaluated in vivo for 96h. SETX expression was downregulated up to 50% in the brain, however, silencing in the liver was variable over time. A study evaluating the effect of SETX silencing on AAV-mediated transduction in vivo using an AAV able to transduce both the liver and the brain after systemic injection is ongoing.
In summary, SETX is a restriction factor for rAAV expression in vitro and a potential target to increase gene therapy efficiency.

P070

Safety evaluation of a novel gene therapy candidate for adrenomyeloneuropathy (SBT101) in nonhuman primates

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Adrenomyeloneuropathy (AMN) is a progressive neurodegenerative disease primarily affecting the spinal cord and is caused by pathogenic variants in the adenosine triphosphate-binding cassette sub-family D member 1 (*ABCD1*) gene. SBT101, an adeno-associated virus serotype 9 (AAV9)-based gene therapy encoding functional human *ABCD1* (h*ABCD1*), is a candidate treatment for AMN that is currently under clinical investigation in a phase 1/2 trial. Prior research in mouse models of AMN has demonstrated reduction in biochemical markers and functional signs of the disease. Here, we present findings from preclinical studies on the safety of SBT101 in nonhuman primates (NHPs) to bridge the gap between rodents and humans. Male cynomolgus macaques received intrathecally administered SBT101 at 7.77E12 to 4.03E13 vector genomes per animal. Biochemical and histopathological analyses were performed over 12 months to assess the reversibility or persistence of treatment effects throughout multiple tissues. Foremost, no treatment-related mortality was observed. Vector genome and transgene expression were maintained at all timepoints evaluated. Non-dose-dependent, minimal to moderate toxicities (axonal degeneration, neuronal necrosis, mononuclear infiltrates) were observed in both target tissues (dorsal root ganglia and spinal cord) and other tissues including heart, kidney, liver, and muscle at month 3 and month 6. These findings had resolved by month 12. Overall, SBT101 was well tolerated over 12 months with persistent vector genome distribution, and transgene expression in healthy NHPs, supporting its investigation in the phase 1/2 trial.

P072

Massively parallel screening of barcoded AAV capsid variants for neutralizing antibody reactivity

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Recombinant adeno-associated viruses (rAAVs) are one of the most promising candidates as vectors to deliver therapeutic genes in order to treat genetic diseases in humans. Their potential is attested by the approval of multiple gene therapies over the last decade and the abundance of ongoing clinical trials for a variety of diseases. However, pre-existing neutralizing antibodies (NAbs), due to natural infection with the wild type AAVs early in a patient's life, can prevent rAAVs from efficiently infecting their target tissues. Consequently, screening for NAbs is typically performed during preclinical work in large animals and prior to treatment of human patients, as high Nab titers frequently represent an exclusion criterion. Currently available NAb assays belong to two major categories, namely, 1) cell-based assays that measure NAb inhibition of rAAV transduction in cells, and 2) total anti-capsid antibody assays that measure total antibodies (TAbs), regardless of neutralizing activity. The cell-based assays typically involve the mixing of rAAVs carrying a reporter transgene, usually luciferase, with serum or plasma followed by infection of cultured cells. These assays have formal regulatory approval and are used to determine the clinical enrollment cutoff. Still, the fact that they are very time- and labor-intensive renders them impractical for (pre-)clinical studies, especially when screening of multiple rAAV capsids, natural or engineered, common or novel, against multiple samples is required. Accordingly, there remains an urgent need for improved assays that enable the rapid, parallel and standardized screening of multiple AAV capsid isolates against multiple NAb-containing samples.

To fill in this gap, we have devised a novel protocol that combines two methods, one traditional and the other next-generation, *i.e.*, cell-based NAb assays and high-throughput parallel screening of barcoded AAV libraries, respectively. For proof-of-concept, libraries of barcoded natural AAV capsid variants were mixed with NAb-containing samples and then used to infect cells. The inhibition of transduction (TI) was assessed on the DNA and RNA levels using next-generation sequencing (NGS) and bioinformatic analysis, allowing the determination of a serological profile for the entire library in a single step. To facilitate a head-to-head comparison of our high-throughput NAb NGS assay with a traditional NAb assay using reporter viruses, we also performed our novel screening protocol using luciferase-expressing barcoded rAAV libraries, to validate the reproducibility of data between the two assays and thus support the robustness and adaptability of our new protocol.

In summary, our assay allows the massively parallel evaluation of NAbs against AAVs and therefore represents an expeditious and cost-effective alternative, which may complement or replace classical methods, especially as the number of novel AAV variants continues to grow at an exponential rate.

P073

BI-GT-101 mediates enhancement of AAV uptake and transduction efficiency

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The need for high AAV doses to achieve clinical efficacy remains a key challenge to AAV utility in the clinic. High vector dose increases cost and has been majorly implicated in the activation of immune responses directed against the AAV capsid, as well as the therapeutic payload. Hence, numerous efforts are currently directed at improving AAV transduction efficiency. In this study, we demonstrate the potency and efficiency of BI-GT-101, a small peptide, which, upon pre-incubation with AAVs, effectively enhances transduction efficiency by two- to four-fold in multiple cell lines of epithelial and fibroblastic origins. Additionally, BI-GT-101 outperforms other commonly used cell penetrating peptides which have been previously used to enhance AAV transduction. Moreover, we show similar enhancement of transduction for multiple AAV serotypes, suggesting that the mechanism of action for BI-GT-101 is serotype-independent. Mechanistically, BI-GT-101 increased vector DNA-uptake by two-fold, resulting in a corresponding increase in the uncoated vector genomes. Our data reveals BI-GT-101 primarily influences cellular uptake of AAV particles, as we observed no difference in the vector uncoating efficiencies between WT-AAV and BI-GT-101-supplemented-AAVs. Furthermore, genetic incorporation of BI-GT-101 in a transduction-incompetent AAV2 restores transduction to near WT-AAV2 level suggesting BI-GT-101 can also facilitate receptor binding. In conclusion, we demonstrate that combining/supplementing AAVs with BI-GT-101 robustly enhances AAV transduction efficiency, thereby opening new opportunities to reduce vector doses by a factor of two to four. This could be significant in gene therapy applications in compartmentalized environments such as the knee, eye, CNS, and in the inner ear.

P075

Directed evolution of engineered AAV replication proteins

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Adeno-associated virus (AAV) has become the most popular viral vector for therapeutic gene transfer in humans. However, numerous questions surrounding the biology of the wild-type virus persist, restricting the streamlining and upscaling of AAV vector manufacturing. A particularly enigmatic step is the replication and packaging of the recombinant AAV DNA genome, in which the four pleiotropic Rep(lication) proteins play pivotal roles that are only partially understood. Previous research including work from us showed that the Rep proteins of the most studied AAV serotype 2 (AAV2) are compatible with the inverted terminal repeats (ITRs) of several other serotypes. Hence, they can support the replication and encapsidation of an embedded genome, with the notable exception of the AAV5 ITRs that differ significantly in sequence and structure. Nonetheless, it is possible that wild-type Rep proteins, while adapted evolutionarily for the processing of wild-type viral genomes, may only function suboptimally during replication and/or packaging of recombinant AAV DNA. Moreover, they might also perform subpar in combination

with synthetic ITRs that have been engineered or evolved independently, and that may ultimately replace the wild-type AAV2 ITRs which are used in most of the current vectors. Likewise, with the growing number and diversity of AAV capsid variants that were designed or enriched for certain properties, the likelihood concurrently increases that wild-type Rep proteins will no longer mediate an optimal capsid-genome interaction and DNA packaging.

Altogether, these considerations have motivated us to adapt our previously established pipeline for DNA family shuffling of AAV capsid proteins to the Rep proteins as well, with the aims to: 1) improve the production yield of wild-type and novel AAV capsids, 2) reduce the proportion of empty capsids during the production process, 3) enhance the replication and/or packaging of recombinant genomes based on different AAV ITRs, and 4) decode the mechanisms, sequences and structures that underlie Rep-capsid and Rep-ITR interactions. To this end, we have shuffled *rep* genes from a wide variety of naturally occurring AAV isolates, resulting in complex libraries for subsequent interrogation through iterative *in vitro* screening for the aforementioned enhanced properties, using a collection of AAV genomes carrying wild-type or synthetic ITRs as templates for viral DNA replication and packaging. The enrichment of shuffled Rep variants with desired properties is comprehensively monitored at high quality and quantity using next-generation sequencing pipelines, comprising PacBio and Nanopore sequencing, which we have adapted and optimized in parallel for this purpose.

The results of this work are expected to benefit our understanding of natural AAV biology including genome replication and packaging, while also enabling the improvement of the components and processes required for large-scale AAV manufacturing.

P076

VAEprop: A generative machine learning approach for designing high-performing AAV capsids for the non-human primate brain

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Recent developments in generative artificial intelligence (AI) are transforming gene therapy, offering new avenues for designing biological sequences in a controlled, data-driven manner. Central to this endeavor is the challenge of AAV capsid engineering, where the objective is to engineer capsids that significantly outperform wild-type AAVs in transduction efficiency. The complexity of AAV biology and the difficulty of predicting the functional effects of mutations have hindered efforts to engineer improved capsids for therapeutic applications. Despite these obstacles, recent advances in machine learning (ML) have demonstrated promising outcomes. The first approach directly explores protein sequence space using a regression model that predicts capsid properties from sequences. While this method benefits from directly maximizing the target property, it also tends to yield a high rate of false positives. The second approach uses generative AI models to learn from patterns seen in functional AAVs, generating high-quality variants that show improvements over wild-type sequences. However, integrating property measurements into the model remains a challenge.

We introduce VAEprop, a hybrid design method combining the benefits of both ML philosophies. We use VAEprop to design variants that improve transduction efficiency beyond either method alone in vitro and in vivo in non-human primate (NHP) studies. Utilizing a Variational Autoencoder

(VAE), a popular generative model, we integrate a VAE's low-dimensional continuous representation with a regression model, predicting the target property from the VAE's representation of a protein sequence. This creates an embedding mapping similar protein sequences to nearby points, allowing for an efficient search for better capsids. By limiting optimization to low-uncertainty regions, we increase our confidence in the functionality of the designed capsids.

We first rigorously compare our method head-to-head with other established methods in a well-powered fast-feedback in-vitro study. VAEprop is compared to two benchmark methods: sampling sequences from a VAE and direct optimization with a genetic algorithm. VAEprop surpasses both methods in balancing risk and reward. We successfully designed capsids with edit distances of up to 16 from wild-type. Roughly half of them package successfully. In contrast, only a quarter of variants designed by the genetic algorithm were successfully packaged. Furthermore, the top-performing capsids designed by VAEprop transduce HEK 293T cells 16-fold better than AAV9 and between 2-4 fold better than the other baseline methods.

We then show that our method improvement translates to non-human primates (NHP). We employ VAEprop to design a few thousand AAV9 wild-type variants with mutations across the variable loop regions of VP3, optimized for the transduction of the central nervous system (CNS). We measure the performance of these capsids in multiple NHPs and show that VAEprop's designs can improve on a high-performing CNS transducing variant previously designed by an earlier generative model.

Our method is an accessible generative AI approach with advantages drawn from two previously distinct AI philosophies. Importantly, we push beyond the in-silico validation standard and provide direct evidence in-vitro and in-vivo that our model can design capsids with field-leading potential.

P077

Novel AAV-vector selected from randomized AAV-libraries efficiently transduce human smooth muscle and endothelial cells

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Gene therapy could become a promising treatment option for cardiovascular diseases that massively contribute to global mortality, but also for rare monogenetic disorders affecting the vasculature like the Marfan syndrome. Adeno-associated virus (AAV) vectors indicate impressive results as a tool for transgene delivery and have led to FDA-approved treatments and a variety of clinical trials are ongoing. Optimizing the AAV-capsid could mediate improved transduction of vascular cells (endothelial cells and smooth muscle cells), minimize off-target effects, and thereby reduce vector doses for treatment. In this study, a random heptamer insertion into the AAV-capsid gene displayed on the vector surface allowed to produce high-diversity random AAV-libraries of the serotypes AAV5 and AAV9. After serial selections by reinfection of murine vascular smooth muscle cells (SMC), deep sequencing on gDNA and RNA levels identified enriched capsid-variants

with potentially improved transduction and gene expression properties. Individual barcoding of the most promising capsid-variants allowed for parallel validation *in vitro*. We identified a novel AAV9-capsid variant (AAV9SMC) which allowed expression of a barcoded reporter gene in murine SMC 6-fold more abundant than AAV9 wild type. AAV9SMC and other promising variants were individually tested with an enhanced yellow fluorescent reporter gene in human vascular SMC and endothelial cells (EC) at different multiplicities of infection (MOI). Flow-cytometry indicated almost 98.9% transduction of primary human vascular SMCs at a MOI of 10^5 and 87.6% at a MOI of 10^4 compared to 0.7% with AAV9 wild type. Transduction of human umbilical vein ECs was also increased (46.7% versus 0.7% with AAV9 wild type, MOI 10^4). Further analyses in small and large animal models are currently ongoing to fully assess the translational potential.

P078

Nonclinical pharmacology of KB408, an HSV-1-based vector designed for the treatment of alpha-1 antitrypsin deficiency, in the *SERPINA1* knockout mouse model

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Alpha-1 antitrypsin deficiency (AATD) is a rare autosomal co-dominant inherited genetic disorder resulting from mutations in the *SERPINA1* gene encoding alpha-1 antitrypsin (AAT). AAT, a secreted α1-glycoprotein, is the most abundant circulating serine protease inhibitor whose principal substrate is neutrophil elastase (NE) in the lungs. AAT's primary function is to bind to and inhibit NE, thus protecting the lungs from unregulated NE activity which can result in parenchymal damage and loss of respiratory function. Despite causing both severe lung and liver pathology, lung disease is of the greatest clinical importance for most AATD patients due to progressive pulmonary impairment leading, eventually, to respiratory failure. Augmentation therapy, consisting of weekly intravenous infusions of plasma-derived AAT, remains the only FDA approved therapy for AATD. However, its clinical efficacy in preventing progressive lung dysfunction is debated, and novel treatments targeting AATD pulmonary disease are needed. To this end, KB408, a replication-defective herpes simplex virus type 1 (HSV-1)-based gene therapy vector encoding full-length human AAT, was engineered for the treatment of AATD-related lung disease. Preliminary data indicated that KB408 efficiently transduced multiple human cell types in culture, including clinically relevant primary human small airway epithelia cells, resulting in secretion of full-length human AAT (Artusi *et al*, ESGCT 2021). Here, AAT was found to irreversibly bind recombinant human NE, supporting bioactivity of the vector-derived human protein. When administered via inhalation to both healthy and *SERPINA1* knock-out immunocompetent mice, KB408 effectively targeted the respiratory tract, promoting secretion of human AAT into both the serum and lung lining fluid, consistent with trafficking of the expressed protein from transduced lung epithelium into the circulation via the interstitial space. To compare relative exposure in different airway compartments based on route of administration, a head-to-head comparison of human AAT exposure in lungs and fluids of *SERPINA1* knock-out mice post inhalation of KB408 versus tail vein injection of plasma-derived human AAT was conducted. Inhaled KB408 therapy resulted in higher concentrations of AAT on the lung surface as compared to a surrogate animal dose of intravenous augmentation therapy, with similar lung tissue exposure levels. KB408 was also found to be well tolerated in the airways of both healthy and *SERPINA1* knock-out immunocompetent mice. Taken together, these observations support the development of KB408 as a novel gene therapy for the treatment of AATD-related lung disease.

P079

DoE as a powerful tool for de-risking AAV process scale-up

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One of the main challenges in bringing AAV-based therapies to patients is the ability to efficiently translate bench-scale processes to commercial GMP manufacturing while meeting the high dosage requirements. To overcome this challenge, we have partnered with Alexion and Polyplus® SA for the development of a highly scalable AAV8 manufacturing platform with a capacity ranging from 1L to 2000L bioreactor scale (1000L scale). In order to accelerate the development and de-risk the scale-up, design of experiment (DoE) approach was used to assess the impact of some essential process parameters such as cell density at transfection, transfection mix volume and Power/Input (P/V) on process performance indicators, such as viral productivity and full/empty ratio at 1L scale. This DoE has helped gain better understanding of the process, especially scale-dependent factors, thereby allowing us to succeed right-first time in AAV8 process scale-up. As a first step of the scale-up, the AAV8 production process was successfully performed at 40L scale in an Eppendorf BioBLU® 50c yielding a titer of $6.98x10^{11}$ vg/ml, which was in the range predicted by the DoE model (7.21×10^{11} +/- 3.39×10^{11} vg/ml). Consequently, a scale-up of the process to Allegro Pall STR200L and STR2000L was undertaken. Consistent process performance and high titer was obtained at both scales, $6.9x10^{11}$ vg/ml for STR200L and $8.2x10^{11}$ vg/ml for STR2000L. To sum up, we have leveraged from DoE approach to de-risk AAV process scale-up, resulting in conserved process outcomes at all scales.

P080

AAV-mediated brain overexpression of a trophic factor improves the pathological symptoms of Rett mice

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Rett syndrome (RTT) is a devastating neurological disorder mainly caused by loss-of-function mutations in the *MECP2* gene. Currently, there is no cure for RTT and the pharmacological treatments available only moderately reduce the severity of the symptoms. In this context, restoring MeCP2 function by Adeno-Associated viral vector (AAV)-based gene therapy holds great therapeutic potential. Nevertheless, the translatability of this strategy is limited by the need for an AAV that can spread widely throughout the brain, restoring MeCP2 at the correct gene dosage in both neurons and glia. To overcome these significant hurdles, we conceived an alternative gene therapy approach based on the overexpression of a transgenic trophic factor (herein TrF). Growth factors play in fact a key role in nervous system development, maturation and functions, and their alterations can lead to several neurological and non-neurological dysfunctions.

The association between RTT and growth factors has been well-established since almost 20 years. In brief, MeCP2 positively regulates the expression of these factors and, in line with this, reduced

levels of numerous growth factors in RTT models have been observed. Many of the brain alterations found in RTT patients are in fact considered to be strictly associated with the general reduction of these pleiotropic molecules. Unfortunately, many growth factors are unstable and poorly cross the blood-brain barrier (BBB), making them unsuitable for conventional pharmacological administration. For this reason, we established a gene-based approach for a chronic and diffuse expression of selected candidates throughout the brain. In particular, we packaged the coding sequence of one TrF into AAV for its delivery directly into the brain. In particular, we exploited the AAV-PHP.eB for its great capability to cross the rodent BBB, and cloned the tNF downstream to an astrocyte-specific promoter. Astrocytes naturally produce and secrete several small molecules, including growth factors that are crucial for neuronal homeostasis. Additionally, these cells are efficiently targeted by both standard (e.g. AAV9) and engineered AAVs variants, making them an optimal cellular target for the overall goal of this gene therapy approach.

In brief, we initially confirmed the capability of astrocytes in culture to produce and release the growth factors after AAV infection by using both Western Blot and Elisa assays. Following this preliminary observation, we treated wild-type and Mecp2-KO mice at neonatal stage by systemic injection with the AAV-TrF, comparing three different doses. Interestingly, both the higher and the intermediate doses caused a dramatic weight loss soon after the injection that we found associated with a strong reduction in blood glucose concentration. Conversely, with the lower dose, animals reached adulthood without adverse effects. At this stage, we observed that Mecp2-KO mice treated with the AAV-TrF significantly improved their motor behavior in comparison with control-treated animals. Effects of the treatment on neuronal morphology, maturation and gene expression will be presented. In conclusion, we have devised a novel gene therapy strategy that does not require neuronal expression and overcomes gene dosage effects, making it suitable for RTT and potentially for many other neurological disorders.

P081

AAV based lncRNA and factor 8 gene therapy is therapeutic in a murine model of hemophilic arthropathy

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Hemophilic arthropathy (HA) is a debilitating joint disease with chronic pain, disability, and poor quality of life in patients with hemophilia. A therapeutic strategy targeted at molecular mediators involved in the disease pathogenesis is crucial to prevent progression of joint damage. We have earlier documented the role of a long non-coding (lnc) RNA nuclear enriched abundant transcript (*Neat1*) in chronic HA, where it positively regulates the expression of matrix metalloproteinases (MMPs) and leads to cartilage degeneration. Based on these findings, we hypothesized *Neat1* downregulation in tandem with factor (F)8 gene therapy could be beneficial for prevention of joint damage in hemophilic arthropathy. To test this hypothesis, we designed a short hairpin (sh)RNA targeting murine *Neat1* and packaged them in Adeno-associated virus (AAV) serotype 5 for efficient delivery to the cartilage tissue. AAV5WT-Neat1 shRNA vector was administered intra-articularly at a dose of $2.5X10^{11}$vgs/leg alone or in combination with a systemic injection of capsid modified AAV8 (K31Q) vector carrying B domain deleted F8 gene (F8-BDD-V3, a kind gift from Dr Nathwani, UCL) ($1X10^{11}$vgs/mice) in a mouse model of chronic hemophilic

arthropathy. FVIII-specific clotting assay revealed increased FVIII activity in treated mice (16% to 28%) after 52 days of vector administration. *Neat1* was significantly downregulated in injured joints of mice receiving only AAV5WT-Neat1 shRNA (16 fold vs. untreated injured joint) as well as in mice receiving both F8 and Neat1 shRNA vectors (40 fold vs. untreated injured joint). Immunohistochemical analysis of injured joint tissues showed that the knockdown of *Neat1* resulted in a reduced expression of MMP3, MMP13, and the inflammatory mediator cPLA2 in injured joints from combination therapy group as compared to the untreated injured joints. Gross examination of joint tissue showed evidence of bleeding in the injured joint that received no treatment whereas bleeding in the joint space was resolved in mice that received either F8 or combination therapy. A detailed histological assessment of the joint sections demonstrated joint recovery in mice treated with both Neat1 shRNA and F8 vectors and comparable to the control joints. This was confirmed by a significant reduction of synovial hyperplasia and villi, absence of hemorrhage or newly formed vessels, minimal amount of hemosiderin, and intact articular cartilage. Our findings indicate that AAV mediated *Neat1* knockdown in conjunction with F8 gene therapy could be a promising approach towards HA treatment. Since *Neat1* and associated pathways are conserved, this could be utilized for treatment of other joint diseases like rheumatoid arthritis and osteoarthritis.

P082

Development and *in vivo* validation of a barcoding method enabling accurate and single-cell level biodistribution

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Adeno-associated virus (AAV) vectors are the most efficient tools for *in vivo* gene transfer and have already showed their potential for the treatment of multiple monogenic disorders. However, limited efficacy in particular in some tissues and the risk of overloading off-target tissues remain, to date, the key challenges to overcome in order to increase potency and safety of AAV vectors.

Currently, many laboratories are focusing their research on capsid engineering aiming to improve the specificity and efficiency of tissue targeting, de-target unwanted tissues and decrease immune response. Engineering methods are often based on the generation of millions of capsids variants followed by rounds of selections, consequently, they require library approaches and subsequent next generation sequencing to be characterized. Moreover, since novel capsids are usually screened in mouse, investigate their efficiency in large animal model close to human is essential.

Here, we developed a barcoded method to assess the *in vivo* efficacy of new engineered capsids in mouse and non-human primate (NHP). We first selected 10 potential barcode DNA sequences of around 80bp, here named QRcodes, and cloned them in a transgene expression cassette between the transgene stop codon and the polyA signal, subsequently packaged in multiple AAV vectors. *In vitro* and *in vivo* experiments allowed us to isolate 7 DNA sequences with no impact on transgene expression *in vitro*. Mice were then injected with single or combined vectors, and AAVs biodistribution and transgene expression on different tissues was assessed by qPCR using QRcode-specific probe sets. As expected, we obtained different biodistribution at genomic and RNA levels, but no significant difference when the vectors were administered alone or in combination. The large size of QRcode DNA allowed us to perform *in situ* Hybridization to analyze

transgene expression of the different AAVs at single cell level. Finally, the QRcode method was validated in NHP co-injected with 7 QRcoded AAV capsids. The analysis of biodistribution and transgene expression in different tissues confirmed the reliability and the robustness of the method for the characterization of new AAV vectors. In contrast with classical barcoded library approaches, the QRcode method also allows to assess the vector biodistribution of selected AAV variants by droplet digital PCR thus providing high-quality biodistribution for multiple capsids in a smaller number of primates.

P083

An AAV-based RNAi and CRISPR vector system to multilaterally combat chronic HBV infections

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Chronic infections with the hepatitis B virus (HBV; CHB) remain a major global health burden, especially since only preventive but no curative treatments are available, leading to 800.000 associated deaths per year. Fortunately, our growing knowledge of HBV biology allows to envision complementary strategies to combat viral infection and to implement a cure for CHB.

Viral replication and protein expression are governed by the formation of low levels of covalently closed circular DNA (cccDNA). While endogenous mechanisms fail to eliminate these nucleic acids, a combination of CRISPR/Cas9 and shRNA-based RNAi may allow to remove cccDNA and to ablate protein expression. We employed our in-house multiplexing technique called TRISPR to combine gRNAs and shRNAs targeting HBV cccDNA or mRNA, respectively. These cassettes were packaged in Adeno-associated viruses (AAV) and initially tested *in vitro*, which showed a predominant effect of the RNAi component. At seven days post-transduction, HBe antigen (HBeAg) levels in supernatants of HepG2-H1.3 cells treated with an AAV-TRISPR vector encoding a non-targeting shRNAs (scrshRNA) decreased by up to 40%, or up to 13.1% in HepG2-NTCP cells. In contrast, a TRISPR construct with the targeting shRNA mediated up to 75% HBeAg reduction in HepG2-H1.3 and 82.5% in HepG2-NTCP cells, respectively. This high effectiveness was corroborated by qPCR-based measurements.

Another challenge towards a cure for CHB is posed by the insufficient immune response against HBV. This is evidenced by an enhanced expression of inhibitory immune checkpoints (ICP) on HBV-specific CD8+ T cells. Knocking down the ligands of these ICP by CasRx may help to reconstitute T cell responses. We therefore screened gRNAs targeting Gal9 or PD-L1 mRNAs and tested them *in vitro*. Surprisingly, knockdown efficiencies were moderate compared to a non-treated (NT) control, or even increased in target expression. Still, we identified two gRNAs in THP-1 cells that reduced Gal9 (hGal9) expression by around 30%, as compared to the scrgRNA control. In NIH/3T3 cells, one gRNA gave a knockdown efficiency of 45%. To assess whether AAV transduction had triggered a reverse effect on ICP protein expression, gRNA knockdown efficiencies were validated by Luciferase reporter assay. Strongest reductions in *Renilla* luciferase signal were observed for human-specific gRNA3 with 86.7%, gRNA6 with 82.6% and gRNA8 with 66.7%. The mouse-specific gRNA2, gRNA4 and gRNA5 decreased the signals by 72.3%, 63.7% and 67.3%, respectively.

The comparison of both assays implies that AAV transduction triggers overexpression of ICPs. The underlying mechanisms remain unknown but may involve TLR9-mediated recognition of the

transgene, which we aim to evaluate through co-expression of TLR9 decoy sequences. Until then, we consider our system as a powerful and promising tool and effort to combat CHB.

P084

Engineered AAV capsids with significantly improved muscle and CNS tropism plus reduced liver and DRG expression compared to AAV9

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Adeno-associated viruses (AAVs) are emerging as important tools for treating diseases of the CNS and muscle. The current generation of AAV-based medicines require high doses when delivered intravenously, which can result in adverse events due to high exposure to the liver and dorsal root ganglion (DRG), as well as increasing the cost of goods. We previously reported the discovery of a VR1 variant (Mut1) that causes a several thousand-fold decrease in liver expression compared to AAV9. Using rationally designed screens of peptide insertions in VR8, both in the presence and absence of Mut1, we identified AAVs with significantly increased tropism for either the CNS or muscle in combination with significant detargeting of the liver and DRG in non-human primates.

Our lead muscle capsids feature RGD-containing 7-mer peptides that improve muscle expression at least 100-fold compared to AAV9. We have identified myopeptides that, compared to AAV9, can cause increased expression in skeletal and cardiac muscle, cardiac muscle only, or skeletal muscle only. In clonal studies in non-human primates, we were able to not only confirm increased muscle and reduced liver expression, but also reduced expression in DRG and reduced incidence of increased liver enzymes compared to AAV9.

Our lead CNS capsids contain peptides that improve tropism in the brain ~250-fold compared to AAV9, with reduced expression in the liver and DRG.

In summary, our novel capsids have the potential to use lower doses than AAV9 to effectively deliver therapeutic cargo to target tissues while relatively sparing off-target tissues such as DRG and liver, thus possibly improving their safety profile. These properties, together with their acceptable manufacturing yields and levels of pre-existing immunity, support further exploration for the promise of these capsids for future medicines.

P085

Characterization of a novel capsid in the central nervous system across multiple species

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Recombinant adeno-associated virus (rAAV) mediated gene delivery is widely pursued as a delivery vehicle for CNS-targeted gene therapy. One major optimization is to improve capsid biodistribution and enhance transgene expression. Towards this goal, we characterized a novel capsid AAV-PRV. We conducted several *in vitro* and *in vivo* studies across multiple species using our AAV-PRV capsid with eGFP as the reporter. We observed stronger GFP expression following transduction of AAV-PRV-eGFP than AAV9-eGFP in human neuroblastoma cells and validated these results in mouse neuroblastoma cells. We then compared the biodistribution and transduction of AAV-PRV-eGFP versus AAV9-eGFP *in vivo* across multiple species, including mice, gottingen minipigs and non-human primates (NHP). In mice, the two viruses were dosed via intracerebroventricular (ICV) injection, with necropsy at 1-month. Using qPCR analysis, AAV-PRV-eGFP demonstrated a significant increase in vector genome distribution and eGFP mRNA expression in various regions of brain and spinal cord. To demonstrate clinical translatability, we then tested these capsids in NHP and minipigs by intracisternal magna (ICM) injection. We evaluated minipig brain and spinal cord tissues and observed more than a 10-fold higher biodistribution in brain and spinal cord with AAV-PRV-eGFP compared to AAV9-eGFP. Immunohistochemistry (IHC) data showed robust eGFP expression with AAV-PRV-eGFP in the brain. In NHP, at 1-month after virus administration, we observed higher biodistribution for AAV-PRV-eGFP than AAV9-eGFP in spinal cord. Although the vector genome distribution for both capsids was similar in different brain regions, IHC results revealed that AAV-PRV-eGFP is stronger at transducing certain cell types in the brain compared to AAV9. Our *in vivo* data across species indicated interesting differences in biodistribution in various brain regions, and AAV-PRV demonstrated particularly robust biodistribution in the spinal cord across species.

P086

Suppression of detrimental transgene expression by optimized artificial miRNAs strongly increases AAV vector yields in HEK-293 cells

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Adeno-associated virus (AAV) vectors represent one of the most attractive platforms for gene delivery in both, preclinical research and therapeutic applications. Traditionally, AAV vectors are produced in human embryonic kidney (HEK) 293 cell lines by transient triple-transfection of plasmids, harboring adenoviral helper genes, AAV-derived replication (*rep*) and capsid (*cap*) genes as well as the transgene expression cassette. As many AAV-based studies use constitutive promoters that are active in HEK-293 cells (e.g., CMV, CAG promoters), the transgene to be packaged is already expressed during the production process. In some cases, for example in exploratory research, where transgene effects tend to be poorly characterized, their expression might exert cytotoxic or antiproliferative effects on the producer cell line, thereby leading to

reduced AAV yields. Transient silencing of transgene expression during AAV production by, e.g., naturally occurring miRNAs or artificial riboswitches has therefore been explored in the field, leading to varying degrees of AAV yield improvements observed in respective studies.

To establish an improved silencing strategy aiming for universal applicability, most efficient transgene suppression, and lowest impact on AAV functionality, we constructed artificial miRNAs based on an optimized miRNA backbone and a miRNA selection strategy that comprised various sources, including a set of >750,000 in silico predicted sequence candidates. Following miRNA candidate assessment in a reporter assay, one selected candidate was tested in the context of AAV production. Expression of the miRNA was either ensured by transfection of a fourth plasmid or – as a novel approach – by insertion of the miRNA sequence into the 3'-untranslated region (3'UTR) within the helper or AAV rep/cap plasmid.

Our results demonstrate that co-expression of the miRNA from either the 3'UTR of the adenoviral helper gene VA or the AAV cap gene not only allowed to retain the traditional triple transfection protocol, but also efficiently silenced cytotoxic transgene expression, thereby increasing AAV vector yields up to 70-fold and 120-fold, respectively. Due to the small size of 22 bp, the miRNA target site can be included multiple times within the 3'UTR of the transgene cassette, thereby only minimally reducing the limited AAV genome coding capacity. In summary, our data show that expressing an optimized, artificial miRNA from the 3'UTR of conventional AAV production plasmids is a highly efficient and universally applicable approach to suppress detrimental transgenes during AAV production in HEK-293 cells, leading to so far unprecedented increases in respective AAV vector yields.

P087

Scalable recombinant adeno-associated virus vector production in insect cells using a single baculovirus construct

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Vectors based on adeno-associated virus (AAV) have emerged as the leading candidates for gene therapy applications to treat genetic diseases. However, industrial-scale production of recombinant (r)AAV remains challenging due the unavailability of a flexible and efficient production platform. The baculovirus/insect cell expression vector system (BEVS) provides a solution, due to its compatibility with scalable productions. For rAAV production with the BEVS, three transgenes need to be supplied: Capsid, Replicase and an ITR-flanked (therapeutic) gene of interest. Several options are available for the introduction of transgenes into the baculovirus genome; the two most well-known being 1) homologous recombination (HR) during co-transfection of bacmid DNA with a donor plasmid in insect cells and 2) Tn7 transposon-mediated transposition in a bacmid in *E. coli*. Both approaches use the polyhedrin locus for transgene insertion, therefore HR and transposition cannot be applied in the same system. Questions arise on what method is preferred for the introduction of AAV transgenes in the baculovirus genome, HR or transposition, and how the method of insertion and the locus affect the yield and quality of the resulting rAAV vectors. Here, we present a bacmid that is compatible for HR (at the polyhedrin locus) as well as for transposition (at the odv-e56 locus). A partial deletion in ORF1629 makes the bacmid compatible for transgene insertion through homologous recombination, while also rendering non-recombinants replication defective. The Tn7

transposition site was introduced at odv-e56 through lambda red recombineering in *E. coli*. The AAV transgenes were inserted in various configurations and the resulting baculoviruses were used for rAAV production in Sf9 insect cells. Baculovirus harvests were characterized by transgene presence and expression, and rAAV capsid and genome titre determination. This work gives insights to the efficient setup of a scalable and stable rAAV production system with a single baculovirus.

P088

Identification of tissue specific AAV variants by highly diverse peptide insertion library screening in mouse and NHP models

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Several procedures have been investigated to create adeno-associated virus (AAV) vectors that target different tissues with high specificity and efficiency. Improving on-target and reducing off-target cell transduction was enabled when peptide ligands were inserted into variable regions (VR) of the AAV capsids. We designed a plasmid library containing 6000 different peptides, mostly derived from a machine learning approach, inserted in both VR-IV and VR-VIII leading to a theoretical diversity of 3.6E+07 variants. Large scale replication-competent virus production was performed in Pro10 suspension cells and next-generation sequencing (NGS) confirmed diversity in the virus by detection of 99.9% of the expected peptide sequences in both VR indicating no loss of certain insertions during production. Subsequently, our AAV library was injected intravenously into C57BL/6 B6N mice (n=6) to track the specificity and efficiency of variants *in-vivo*. DNA samples from several tissues including heart, brain, liver and others were applied to polymerase chain reaction (PCR), generating amplicons covering both VR. Initial Nanopore sequencing of samples from one mouse allowed identification of brain and muscle (quadriceps) specific AAV variants. Ongoing deep sequencing (Illumina) followed by subsequent bioinformatic analyses will further enlighten our understanding of tissue specificity. Considering the outcomes, injection of the same AAV library into non-human primates (NHP) will be carried out to monitor AAV variants in a large animal model.

In summary, we have, and continue to screen, a highly diverse peptide insertion AAV library *in-vivo* to identify tissue specific and efficient AAV variants with the goal of enabling improvements in gene therapies.

P089

Substantially improved gene transfer to interneurons with second-generation glutamate-receptor targeted DART-AAVs

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Adeno-associated viral vectors (AAVs) are a widely used platform for gene transfer in neuroscience research. Applications include genetic delivery of reporters, sensors and effectors e.g. for gene function studies and disease models. Although naturally occurring serotypes can have preferences for certain tissues, selectivity for particular cell types including those of the CNS has not been observed. Still, many applications would benefit from cell-specific transduction. To meet this need, our lab designed an AAV2 presenting a designed ankyrin repeat protein (DARPin) at the N-terminus of the VP2 (AAV-VP2N). This approach was used to target parvalbumine positive (PV+) interneurons, which play a role in different neurological diseases, via GluA4, a surface receptor highly expressed on PV+ cells. AAVs presenting the 2K19 DARPin targeted to GluA4 transduced PV+ cells with high specificity, but exhibited moderate transduction efficiency.

Here we characterize two new configurations of targeted AAVs presenting the 2K19 DARPin in the GH2/GH3 loop of either VP1 (AAV-VP1L). These were compared to the first-generation AAV-VP2N. The second-generation particles were consistently produced at approx. 2-fold higher genomic titers than AAV-VP2N and displayed the expected particle characteristics when analysed by Western blot and electron microscopy. As revealed by particle ELISA, AAV-VP1L bound soluble GluA4 protein more efficiently than AAV-VP2N, indicating better DARPin surface accessibility . This was consistent with gene transfer activities. Stocks of AAV-VP1L displayed 18-fold higher functional titers than AAV-VP2N on GluA4-overexpressing A549 cells and did not transduce A549 wt or A549-AAVR-ko cells. This high gene transfer efficiency was confirmed when AAV-VP1L and AAV-VP2N were compared for reporter transfer into rat hippocampal organotypic slice cultures. AAV-VP1L transduced around five-fold more cells reaching approximately 60% of all PV+ cells. Guided by the same DARPin, both vectors showed similarly high specificities for interneurons. Despite the substantially higher transduction efficiency of AAV-VP1, electrophysiologic characteristics of transduced cells remained unaltered and confirmed the transduction of a heterogeneous group of interneurons.

Enabling highly efficient transduction while retaining the specificity and low toxicity of the first-generation vector, DARPin insertion into the GH2/3 loop represents a major advance in receptor-targeting technology. The modular DARPin-targeted (DART-) AAV platform characterized here, allowing a convenient exchange of the binder, will facilitate a wide range of applications in neurobiology and beyond.

P090

Production of AAV vectors using synthetic, enzymatically produced linear DNA

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Adeno-associated virus (AAV) remains a popular vector for gene therapy. However, AAV manufacture faces several challenges to keep pace with the demand for large scale, high quality batches. The standard plasmid triple-transfection presents several issues including availability and cost of GMP grade plasmid, sequence fidelity of the inverted terminal repeats (ITR), and safety concerns over non-specific encapsidations. 4basebio has developed a proprietary, scalable 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. Here, we compared the production of AAV vectors using hpDNA™ encoding for the typical Adenovirus helper functions, *rep* and *cap* genes, and an expression cassette consisting of AAV2 ITRs and a reporter gene driven by an ubiquitous promoter. Conventional plasmid triple-transfection was used as a control. We achieved equivalency in viral genome titres, full:empty ratios and infectivity between the two production methods. We have demonstrated that functional AAV vectors can be produced using hpDNA™, which could greatly accelerate therapeutic development of gene therapy programmes. The technology could overcome the difficulties associated with complex ITR structures required for AAV production, which are inherently difficult to synthesise via bacterial propagation systems. Moreover, the lack of plasmid backbone sequences such as antibiotic resistance genes enhances the safety profile of the AAV product.

P091

Enhancing yield and safety of adeno-associated virus (AAV) vectors via molecular optimization

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Recombinant adeno-associated virus (rAAV) vectors are promising tools in gene therapy, offering demonstrated strategies for treating diverse genetic diseases. However, their extensive translation to commercially treatments is hampered by challenges in scalable production and potential safety concerns when administered at high doses. We have developed an innovative strategy to address these barriers in AAV vector production through a variety of molecular enhancements.

We introduced systematic modifications into the AAV rep/cap and adenovirus helper plasmids, by removing, modifying, and incorporating novel genetic elements designed to boost vector yield. Enhancements include the use of codon modification to enhance gene expression cassettes, and the optimization of promoters and transcription regulatory sequences to enhance gene expression. Simultaneously, we removed or reduced viral genes that were found to be non-necessary or overexpressed. One important innovation from this work is the development of an adenovirus (Ad) helper plasmid for AAV production that is reduced in size to under 7kb, which is approximately 5kb smaller than currently used Ad helper plasmids. This smaller Ad helper

plasmid has improved manufacturability and minimized potential biosafety risks associated with residual adenovirus DNA or proteins.

To assess the impact of our modifications, we compared the modified plasmids to their traditional counterparts in terms of both AAV vector yield and product quality. Our findings demonstrate a significant increase in vector titers, showcasing the potential of this approach for improving the scalability of AAV vector production. Furthermore, vector characterization assays exhibited equivalent or improved attributes, affirming the quality profile of the AAV vectors produced with the improved expression plasmids.

Our research opens up new avenues for improving the production and safety of AAV-based gene therapies. By systematically evaluating and modifying the genetic elements of AAV production plasmids, we have achieved enhanced production yields, facilitating large-scale manufacturing necessary for wider clinical and commercial use. Simultaneously, the improved safety features minimize potential hazards, potentially reducing adverse reactions in gene therapy recipients. This work underscores the potential of thorough evaluation of the molecular reagents used produce AAV and the ability to further advance AAV vector production.

P092

Leveraging the potential of AAV full/empty particle separation on anion-exchange resins by applying mechanistic modeling

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The chromatographic processing of adeno-associated virus (AAV) is an increasingly important and much-debated challenge. The overarching goal is to separate full and empty AAV particles with high yield and purity. Currently, the industry is exploring various methodologies to address this off-platform challenge, with a focus on straightforward scalability, robustness, and efficiency. However, the existing approaches often fall short of meeting these targets. Consequently, successful process development for AAV separation currently requires time-consuming experiments, large sample volumes, and distinct analytical methods.

Chromatography is one way to tackle this challenge. Anion-exchange (AEX) resins provide a scalable solution relying on the differences in surface charge of full and empty particles, which makes them separable based on ionic strength. Elution of the AAV particles is typically induced via salt steps. The specific AAV species' retention time depends on their characteristics and the influence of the surrounding environment like buffer composition and the use of buffer additives such as $MgCl_2$. For successful separation with AEX, the optimization of the separation method and additive concentration are crucial. Mechanistic modeling is the tool of choice to screen the design space for the optimal AEX chromatography method that enables the full separation performance of the resins. However, the implementation of mechanistic models can pose new challenges, especially for inexperienced users. This is partly due to a lack of simple and straightforward modeling and experimental guidance.

We present a workflow showcasing *in silico* process optimization of full and empty AAV5 particle separation on strong AEX resins, such as Capto™ Q ImpRes or Capto™ Q. Only few calibration experiments and analytical assays were necessary to develop a predictive model. Full and empty AAV particles were identified and modeled. *In silico* process optimization allowed adjustment of

the experimental method and the buffer composition for optimal full/empty AAV particle separation, increasing yield and purity of full AAV particles.

We aim to provide reliable and reusable guidance for experimental planning and model-based process optimization for AAV full/empty particle separation, leveraging the full separation performance and straightforward scalability of AEX resins. The guiding workflow we describe for model-based process development with GoSilico™ chromatography modeling software can help process developers meet time-to-market demands. In addition, the workflow can potentially lower the threshold for first-time users to get started with mechanistic modeling.

P093

Quantification of viral transcripts by ddPCR as a novel tool to assess AAV vector productivity

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Adeno-associated virus (AAV) therapeutic vectors have shown great clinical successes in the last decade, however cost-effective manufacturing of high-quality vectors still constitutes a major hurdle for the sector. Improvement in the AAV manufacturing process may be achieved by an increased understanding of the timing and mechanisms of viral particle assembly.

As quantitative assessment of viral proteins in crude cell lysates may be challenging, we designed a multiplex droplet digital (dd) PCR-based method to detect viral mRNA transcripts including Rep78-68, Rep52-40, and Cap from the AAV construct, and E2, E4 from the helper construct. We then tested the assay on a selection of in-house polyclonal and monoclonal high producer and low producer HEK293 cell lines for AAV production in shake flasks (150 mL) and Ambr250 (250 mL) during a time-course analysis.

Our hypothesis is that differential expression patterns of viral genes may have a critical role on the resulting AAV yields. Therefore, a better understanding of limiting expression levels for viral genes may be leveraged to achieve improved productivity. Viral transcripts were detectable at early time points post-transfection and normalised expression kinetics were derived for each gene during the production time frame to identify peaks of expression for each gene. Relative quantification by ddPCR was demonstrated to allow resolution of the expression patterns across cell lines with different productivities. Whilst we observed similarities in the overarching expression kinetics of the viral genes, we noted some expression signatures were correlating with production yields and may need further investigation. Similar gene expression trends were observed in shake flasks, though overall viral gene expression levels were lower, suggesting viral expression patterns might be linked more to the cell line than to the production system.

This assay added a useful tool to further our understanding of viral gene expression during AAV2 vector production and may be used to attribute causative effects of process development changes to AAV productivity outcomes. Novel analytical solutions that enhance our understanding of the biological cues underpinning manufacturing processes have the potential to accelerated process development improvements and in turn bridge current manufacturing gaps in the industry.

P094

Enhancing the efficacy of gene therapy for Duchenne muscular dystrophy through AAV particle coating

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Biologic therapeutics are steadily becoming the new standard in the biomedical field, particularly in the treatment of genetic disorders associated with specific genes, such as Duchenne muscular dystrophy (DMD). Despite extensive research efforts, current treatment options for DMD only focus on managing symptoms. However, a promising approach utilizing gene therapy with adeno-associated viral vectors aims to address the root cause of the disease. Unfortunately, the immune system recognizes and eliminates these vectors before they can perform their therapeutic effects. To overcome this challenge, we have successfully developed a novel solution by coating AAV particles with our proprietary polymer through covalent bonding. These modifications have yielded significant improvements, including enhanced transduction efficiency in various cell lines and improved distribution within muscle tissue in murine models. Moreover, the modified viral particles have exhibited reduced interactions with neutralizing antibodies, thereby enabling the potential for repeated administration of the coated therapeutic AAV particles for systemic treatment. Based on the results observed, we have incorporated a specialized muscle-targeting peptide into our polymer. This strategic modification has resulted in an amelioration in transduction efficiency specifically within muscle tissue.

P095

Rapid AAV pre-formulation screening using a commercially available buffer kit and minimal sample requirement

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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 established a low-volume, high-throughput pre-formulation screening approach that requires only minimal sample amounts and provides results within two days. Starting with only 4 mL of an AAV3-like capsid (2-4E12 vg/mL), we used a commercially available buffer kit containing 96 FDA/EMA-approved biologics formulations to generate thermal (nanoDSF) and colloidal (DLS) stability data.

Using nanoDSF, we showed that thermal stability of this serotype is strongly influenced by pH, whereas other formulation parameters & excipients had negligible effects on AAV melting temperature (T_m). Specifically, thermal stability increased with decreasing pH, with up to +15 °C improvement in T_m compared to the basic formulation of the starting material.

Temperature-induced onset of AAV aggregation (T_{onset}) was determined using DLS in a 384-well plate format within 8 hours. We demonstrated that T_{onset} could be increased by up to +25 °C compared to the starting material and that a minimum ionic strength-level (around 200 mM) is

required for colloidal stabilization of AAV which is in line with published data. Ionic strength was found to be the only major contributor to colloidal stability.

The data provides a solid basis to inform further rational formulation development. The approach is capsid agnostic: it can be applied to any AAV serotype or other novel modalities based on protein nanoparticles.

P096

Quantification of rAAV infectivity using the TESSA™ Rep Enabled AAV titration (TREAT) assay

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Infectious titration of recombinant adeno-associated virus (AAV) vector is a key attribute to enable preclinical development for rAAV-mediated gene therapies and for product release testing of clinical rAAV vectors. However, accurate measurement of rAAV infectious titres for a range of AAV serotypes is challenging due to their low transduction efficiency *in vitro* and associated assay variations. HeLa RC32 cells are most commonly employed for infectious titration of rAAVs using the Tissue Culture Infectious Dose 50 (TCID50) assay. These specially engineered HeLa-based cell line contained stable integration of AAV rep/cap genes to enable rAAV vector replication by co-infection with wildtype adenoviruses. As significant disadvantages, requirements for wildtype adenovirus can cause safety concerns and limitations to use HeLa-based cell lines bring into question how representative the titres are compared to target cell types or tissues.

To enable robust and accurate titration of rAAV infectivity across various target cells, we developed a novel rAAV infectious assay based upon our Tetracycline-Enabled Self-Silencing Adenoviral (TESSA™) system, entitled 'TESSA-Rep Enabled AAV titration' (TREAT) assay.

TREAT employs a self-silencing TESSA™ virus for delivery of adenoviral 'helper' and AAV Rep to enable DNA amplification of the rAAV genome within infected cells for detection of single infection events, and quantification of rAAV infectious titre via the TCID50 assay. We showed that expression of both adenovirus E1 and AAV Rep from TESSA is essential for rAAV genome replication. Amplification of the rAAV genome by the TESSA-E1-Rep virus in permissive cells allowed sensitive detection of infection events using qPCR. TREAT enabled accurate titration of various serotypes of rAAV vectors in a wide range of target cell types, providing a more informative infectious titre for rAAV batch quality assessment, and for research and clinical applications.

P097

Improving adeno-associated vector (AAV) manufacturing bottleneck by unravelling HEK cell biology

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The development and commercialisation of adeno-associated vector (AAV) gene therapies has seen an exponential growth in recent years with a total of 7 products ever approved by EMA or FDA, and more than 500 products in the pipeline from pre-clinical through to Phase II/III. Clinical data from the first generation of AAV therapies has demonstrated promising curative potential in small patient populations with rare and ultrarare diseases and unmet therapeutic needs. For such indications, vector supply needs are generally satisfied by the established AAV industry manufacturing capacity. However, current technologies and processes are not sufficient to meet the ever-increasing viral vector demand, with an estimated need for a 100- to 1000-fold yield improvement to address large patient population with high prevalence diseases.

Several approaches have been undertaken to bridge the viral vectors manufacturing gap, including selection of clonal high producer cell lines, host cell line genetic engineering, design and optimisation of cell culture reagents and expression constructs, development of advanced bioprocessing technologies and modalities, etc. However, the success of these strategies has been typically limited to approximately 10-fold yield increase, which is still below the industry capacity needs.

We hypothesize that larger improvements in AAV productivity and quality may be achieved upon harnessing the biological pathways underpinning manufacturing processes. To this end, Cell and Gene Therapy Catapult is spearheading the creation of an industry-led HEK Consortium to bring together resources and expertise in the sector with the ambition of overcoming the high AAV manufacturing demand. At the core of this initiative there is an unprecedented data-driven programme that generates multi-omics datasets including transcriptomics, proteomics, and metabolomics from scalable AAV production processes. Through the integration of this large omics data, we are starting to map cellular pathways that hinder or sustain viral vector biosynthesis. A comprehensive understanding of HEK cells biological systems will enable us to design and screen for novel engineered cell lines with a more efficient utilisation of metabolic resources towards AAV bioproduction. We anticipate these novel tools will have a high exploitation potential and high impact on the future AAV industry supply.

P098

Nanobody-based adaptor proteins improve the transduction of target cells with different AAV serotypes

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Low cell specificity and high off-target effects remain limitations in the application of AAV gene therapy vectors. Nanobodies, the single variable immunoglobulin domains of camelid heavy chain antibodies, are small, highly soluble, and can readily be reformatted into bispecific formats and other fusion proteins. We have shown that insertion of membrane protein-specific nanobodies into AAV capsid proteins strongly enhances the transduction of target cells expressing the membrane protein (1). Here, we show that transduction of these target cells with AAV vectors is also greatly improved when adding soluble, bispecific nanobody-based adaptor proteins. Such bispecific adaptors consist of a membrane protein-specific nanobody genetically fused to an AAV-specific nanobody via a flexible glycine-serine linker. We selected several AAV-specific nanobodies from immunized LaMice (transgenic mice that carry the llama Ig heavy chain locus) via classical hybridoma technology. We cloned the VHH-coding regions into a eukaryotic expression vector upstream of hinge, CH2 and CH3 domain of rabbit IgG and verified specific binding to different AAV serotypes by immunofluorescence microscopy and ELISA. We fused selected AAV-specific nanobodies to nanobodies specific for a variety of structurally distinct membrane proteins, including transmembrane and GPI-anchored ecto-enzymes. Addition of such bispecific adaptors to target cells expressing the corresponding membrane proteins greatly enhanced transduction of these cells by AAV serotypes recognized by the second nanobody. The modular design of these adaptor proteins allows easy exchange of either nanobodies. We conclude that nanobody-based adaptors are versatile tools to improve AAV transduction of target cells *in vitro* and, if validated *in vivo*, possibly also in the clinic.

1) Eichhoff AM et al. 2019. Nanobody-Enhanced Targeting of AAV Gene Therapy Vectors. Mol Ther Methods Clin Dev. 15:211-220.

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P099

AAV8 trafficking and efficacy in muscle cells of 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. This limitation leads to the use of high doses of AAV and oblige to optimize the strategy to get the best efficiency with lowest dose of therapeutics for clinical use. To be efficient, AAV vectors need to reach cell nuclei by multiple-step events including receptor-mediated endocytosis, intracellular trafficking thought the endolysosomal pathway and nucleus entry in which the vector synthesizes the second-strand of its genome to express the transgene. However, little is known about the AAV fate in diseased muscles. Our project aims to characterize cellular uptake, intracellular transport and expression efficacy of AAV8 in muscle cells from DMD patients. We first analysed whether AAV8 internalisation and expression requires the universal AAV receptor (AAVR) in DMD and control cells. We showed that AAVR is likely involved in intracellular trafficking of AAV8 to the trans-Golgi network and vector maturation. Our study showed that the AAV8 is equally internalized in DMD and control muscle cells while it is less efficient at expressing its transgene in DMD compared to controls. Moreover, immunostaining showed that the vector entire capsids remain stable in the perinuclear region. We further investigated the proper synthesis of the second strand of the viral genome by comparing the transgene expression of a single stranded AAV (ssAAV8-GFP) and a self-complementary AAV (scAAV8-GFP) containing a double stranded genome. Preliminary data suggested that the vector transport and/or nuclear uncoating much more than DNA conversion are responsible for the expression defect in DMD muscle cells.

P100

Nanopore sequencing of recombinant AAV vectors for the characterization of DNA content

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Adeno-associated virus (AAV)-mediated gene therapy has started to prove itself as an advanced, transformative therapeutic modality for treating patients who are suffering from devastating and often untreatable diseases. However, the manufacturing process is complex and does not always yield a pure drug product. Contaminant DNA such as rearranged vector DNA, host cell genomic DNA, or plasmid DNA can be packaged into the viral capsids. Nanopore sequencing allows for careful monitoring and characterization of DNA content because of its ability to analyze long sequences without amplification. The aim of this study was to develop a customized nanopore sequencing pipeline for the analysis of the DNA content of recombinant AAVs. Five different rAAV batches (3 rAAV2, 2 rAAV9) were analyzed. DNA libraries were prepared with the Rapid Sequencing Kit and sequenced with a R9.4.1 flow cell on a MinION. Reads of ≥1,000bp were selected, mapped against the reference sequences with minimap2, and analyzed using a custom script. Additional mappings against the human genome (hg38) and transfer/helper plasmids were performed to check for DNA contaminants. Between 95.0% and 99.6% of the reads mapped exclusively against the rAAV genome with 83.6%-90.8% and 4.9%-12.1% classifying as high- and low-quality, respectively. Between 0.58%-1.81% also mapped to the transgene plasmid backbone, helper plasmid and hg38. The remaining reads, 0.36%-5.15%, mapped solely to the transgene plasmid backbone, helper plasmid, hg38 or were sequencing artifacts. In conclusion, our customized nanopore sequencing pipeline allows for qualitative and quantitative DNA content analysis of rAAV, including contaminant sequences. In a next step, we aim to validate the pipeline to assess the accuracy and precision of the data and determine possible sources of bias.

P101

Papillomavirus as a novel viral vector for gene therapy of Olmsted syndrome

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Olmsted syndrome is a dominant genetic skin disease caused by a point mutation in the S4-5 internal loop of 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 spontaneously active, thus increasing intracellular calcium levels; this leads to hyperproliferation and defects in the maturation of basal keratinocytes, invasion of immune cells, and chronic itch and pain.

Here we have asked whether we can reverse the disease phenotype of Olmsted syndrome by disrupting gene expression in a cell type-specific manner. *In vivo* gene therapy of the epidermis is particularly challenging: AAVs, the most common viral vectors, are not efficient in targeting keratinocytes, while lentiviruses were until now used for skin gene therapy only *ex-vivo* on reconstructed skin because of their ability to integrate into the host genome. We reasoned that we could employ papillomavirus (PV) as a viral vector: it has a natural tropism towards keratinocytes, its double-stranded DNA cargo remains episomal, and it can package up to 8 kilobases - a much larger capacity than AAVs.

We used papillomavirus to encapsidate SaCas9 with a gRNA to knock out Trpv3. 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. We transduced both a keratinocyte cell line and mouse primary keratinocytes with PV-SaCas9. Nearly 50% of the alleles showed indel formation ten days after transduction. Next, we evaluated the functionality of the disrupted gene via FURA-2 calcium imaging. One week after the transduction of PV-SaCas9, keratinocytes showed a significant reduction in their response to carvacrol, a TRPV3 agonist.

Hence, we have demonstrated that papillomavirus can be engineered to efficiently deliver a gene of interest to keratinocytes, resulting in a promising new candidate for skin gene therapy.

P102

Small beginnings, great opportunities – Optimize your AAV production from cell to process

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As adeno-associated virus (AAV)-mediated gene therapy has evolved as the leading platform for in vivo gene therapy, efficient AAV production processes are becoming increasingly important to address additional markets besides rare inherited diseases. High-throughput screening methods are essential to find optimal cultivation conditions and HEK293 clones that provide sufficient yields of high-quality products. As a scaled-down, automated platform, the Ambr® 15 cell culture system allows parallel cultivation of multiple cultures within a controlled environment and screening among the high amount of process parameters, making it ideal for identifying the best

cell clones and setup. The optimized process parameters must then be transferable to the actual production process on a larger scale. In the context of identifying high-producing HEK293 clones, we demonstrate the transferability of screening data from the Ambr® 15 to 2 L bioreactor scale. For this, single cell clones from newly suspension adapted HEK293 cells were cultivated in chemically defined culture medium HEK TF (Sartorius Xell) in shake flask scale. For AAV production, PEI MAX (Polysciences) and PEI Pro (Polyplus) were used for transient transfection with a two-plasmid system for AAV2, and AAV8 (Plasmid Factory) with GFP as GOI. Best performing clones were transfected in Ambr® 15 and the impact of stirring speed, transfection reagent as well as feeding conditions on AAV genomic titre was analysed. While stirring speed had little effect on AAV yield, a genomic titre increase of more than two was achieved by feeding 24 hours after transfection with 10 % HEK FS (Sartorius Xell). The data obtained were representative of subsequent 2-liter bioreactors with very comparable genome and capsid titres, whereas shake flask data often show greater divergence due to unregulated cultivation conditions. Our results show that it is worthwhile to use high-throughput methods at an early stage of clone screening and process development, and that data obtained at an early stage can be used to predict behaviour in regulated systems.

P103

A robust synthetic hepatocyte-specific promoter for therapeutic expression of human coagulation factor IX in AAV-delivered gene therapy for haemophilia B

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Synthetic rationally designed promoters, optimised for expression of a given therapeutic gene, in a specific target tissue, with low off-target expression, and tailored to the viral vector of choice, promise to radically improve transgene performance in gene therapy. We have employed a bioinformatics-informed promoter discovery pipeline to identify champion promoters that are specifically active in human hepatocytes. A critical test of such promoters is the ability to drive effective expression of a therapeutic transgene at physiologically appropriate levels. Using the therapeutically relevant human coagulation factor IX gene, we demonstrate that synthetic promoters can achieve tissue-specific levels of transgene expression that are equivalent or better than currently employed regulatory elements.

Loss or impairment of the human factor IX gene results in, a potentially fatal, inherited monogenic disease Haemophilia B that is characterised by reduced or delayed blood clotting following injury or surgery. Recombinant adeno-associated virus (rAAV)-based Factor IX gene replacement therapy achieves substantial reductions in bleeding episodes for patients in clinical trials. Significantly, an increase in FIX levels to >5% of normal FIX blood concentrations leads to improvements for the most at-risk patients. Therefore, novel promoter designs that achieve near normal, or >40% of normal, blood FIX levels are likely to provide complete cessation of spontaneous bleeding episodes for patients.

Here, we validated the capacity of champion promoters identified from our promoter development pipeline for efficient expression of FIX. Among the successful enhancer-core promoter assemblies, we identified the enhancer-promoter sequence WOSP-HS5 that displays an attractive expression profile. WOSP-HS5 provides very low to undetectable expression of FIX in

293-based AAV bioproduction cell lines, and little if any expression in a range of non-hepatic cell types. By contrast, WOSP-HS5 drives efficient expression of FIX in a range of hepatocyte related cell lines, in human iPSC-derived hepatocytes and in primary human hepatocytes. Moreover, WOSP-HS5 provides FIX levels that are equal to or greater than that obtained from industry standard promoters used for therapeutic delivery of FIX in hepatocytes. We anticipate that further improvements in WOSP-HS5 design, in combination with optimisation of the FIX transcription unit, could push circulating blood FIX levels into the zone required for a robust and sustained therapeutic impact in haemophilia B. Thus, rational promoter engineering can be a potent and effective way of improving performance, efficacy, and safety of viral vectors for gene therapy.

P104

Comparative study of AAVs' transduction efficiency at various developmental stages of the inner ear

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Adeno-associated virus (AAV)-based gene therapy offers promising treatment opportunities for inner ear diseases, including deafness and balance disorders. AAV cell tropism and transduction rate can vary depending on the inner ear stage. So far, no study has been able to characterize AAV tropism for a given serotype at various developmental stages of the inner ear.

We compared for the first time the transduction rate and targeting specificity of two naturally occurring AAV stereotypes, AAV2 and AAV8, and two synthetic ones, AAV9-PHP.eB and AAV-Anc80, expressing Green Fluorescent Protein when administered at different developmental stages.

For this purpose, each recombinant AAV vector was administered into the inner ear of wild-type mice through the round-window membrane at three developmental time points, i.e. embryonic, neonatal, and adult stages.

Our results showed that each injected recombinant vector has a distinct inner ear tropism transducing cochlear and vestibular hair cells, support cells, and neurons at different rates depending on the capsid. Furthermore, the transduction rate and the target specificity of these AAV vectors vary according to the inner ear developmental stages. For most AAV capsids tested, the transduction rate of inner ear cells was maximal at the neonatal stage and reduced at later stages.

P105

The challenge of ITR engineering to improve the production and decrease the immunogenicity of AAV viral vectors

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Recombinant Adeno-Associated vectors (rAAV) are composed of an icosahedral capsid defining its serotype, inside which an expression cassette replaces the viral genome of origin. Only two intriguing terminal sequences, the Inverted Terminal Repeats (ITR) located at both wild-type genome extremities, are essential and sufficient for rAAV production and have to be kept. They represent the only genomic element of origin present in recombinant vectors. Gene therapy using rAAV has allowed incredible clinical successes, however, issues such as vector immunogenicity and production yield still remain to be overcome. To this end, vector engineering enables capsid and genome optimizations to improve safety and efficacy. However, strikingly, ITR optimization has been poorly explored.

For historical and convenience reasons ITR from AAV serotype 2 (ITR2) are mostly used in recombinant vectors in a truncated version of 130 base pairs. They are composed of imperfect telomeres forming a particular T-shape secondary structure and of essential binding sites for replication and encapsidation of newly formed genomes. According to literature and existing studies, both the sequence (Rep Binding Elements, D region) and structure (T-shape) of ITRs are essential for viral life cyle. Their manipulation can be challenging, explaining their relatively low exploration in the scientific community.

Here, we describe a number of 3 ITR engineering based on the literature knowledge in order to (i) improve rAAV production and/or (ii) reduce rAAV immunogenicity. We rationally designed 3 ITR2 mutants and checked for the T-shape persistence by secondary structure prediction using RNAfold tool. We cloned the mutant ITRs on both side of a CMV-GFP cassette in a vector plasmid and ITR integrity was verified by sequencing. Finally, rAAV production was tested by transient plasmid transfection of HEK293 adherent cells. We evaluated replication by vector genome copy number quantification in cell pellets and overall production by vector genome copy number quantification in culture supernatants by qPCR targeting the ITR and the transgene sequences.

Our results show that ITR mutations lead to various unexpected results and highlight how these small sequences can dramatically impact vector production. One mutant did not allow any rAAV production. The two other mutants significantly reduced vector yields before purification, and finally only one vector could be purified by Cesium Chloride gradient and is currently under evaluation. Consequently, even without modifying ITR sequences known to be essential or disrupting their predictive secondary structure, ITR mutations lead to a decrease or a complete loss of rAAV production. Their sequence and structure could possess undescribed features such as unknown co-factor binding or particular flexibility allowing genome replication and encapsidation. This study gives an additional insight into the challenge of ITR modifications and questions the lack of the current knowledge of ITR functions at a time where rAAV products are in the market.

P106

In utero systemic administration of natural and engineered rAAV serotypes enables gene delivery to diverse cell types in the fetal mouse brain

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In utero gene therapy (IUGT) holds promise for addressing disorders in the developing embryo, with early intervention particularly important for congenital diseases. Recent technological advances have enabled genetic access to the mouse fetus by injecting recombinant adeno-associated viruses (rAAVs) into the fetal circulation. rAAVs are well tolerated by the fetal immature immune system and their non-integrating cargo can be customized to deliver transgenes for functional protein expression, gene regulatory elements, and gene editing constructs. However, there are still substantial challenges to overcome to enable effective and safe genetic intervention during early developmental stages. Prenatal systemic rAAV delivery leads to ectopic organ transduction and low expression in the fetal brain, limiting the technology to treatment of multi-organ disease states. While genomic editing in the embryo is advantageous due to the reduced mass and access to progenitor cell populations, transgenic cargo is also subject to dilution as the embryo undergoes rapid cell divisions. In order to advance IUGT toward treatment of prenatal disease, characterization of the gene editing potential of systemically-delivered rAAVs at various developmental stages is necessary. Therefore, we set out to identify rAAV variants with reduced off-target organ transduction and develop delivery technologies for early systemic access using the mouse embryo as a model.

To characterize genetic intervention at an earlier developmental time point than previously reported, we used an intravenous delivery method for systemic access to the mouse embryo as early as embryonic day 12 (E12), when the fetus is roughly 6% the mass of a P0 pup. We injected Ai14-TdTomato reporter mice with an AAV9 virus encoding Cre-recombinase and eGFP linked by a P2A self-cleaving peptide, allowing us to identify cells that are currently transduced as well as those whose ancestors were transduced. Embryos injected at E12 showed broader neuronal coverage of the CNS and higher expression in the periphery than those injected at E15, when assessed at E18. These results indicate that gene manipulation in the E12 embryo with rAAV has potential for efficient gene editing in the brain, but is lacking in organ specificity.

To build a specialized toolbox for systemic delivery to the embryonic brain, we compared the transduction profiles of seven rAAV capsids systemically injected into E15 mouse fetuses. We focused on a diverse array of natural serotypes, published engineered brain-tropic capsids, and unpublished capsids that have shown diverse transduction profiles. Using bulk DNA and RNA sequencing across tissues, we identified the rAAV capsids with the highest brain transduction and lowest ectopic organ expression to be used as a parent serotype in a capsid evolution library. We also identified capsids enriched in the embryonic liver, heart, and lung. Integrating specialized capsids and gene regulatory elements with early systemic delivery may open the path to targeted, efficient in utero gene therapy in mammalian disease models.

P107

KJ103, a novel variant of an IgG-degrading enzyme with a lower titer of pre-existing anti-drug antibodies, which could improve safety, efficacy, and potential of multiple-dosing along with AAV-based gene therapies

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KJ103 is a novel bacterial-originated IgG-degrading enzyme from non-human pathogenic *Streptococcus equi subsp. equi*, that could potentially become a pre-treatment of AAV-based gene therapy by eliminating AAV-neutralizing antibodies (Nabs), for better safety and efficacy, furthermore, expand the treatable population and enable the re-dosing in gene therapy. A randomized phase I was performed on healthy individuals in New Zealand and China. Pre-existing anti-drug antibodies (ADA) in patient were not considered as exclusion criteria. The safety, tolerability, pharmacokinetics (PK), pharmacodynamics (PD), IgG cleaving efficacy, and other exploratory endpoints were evaluated among single doses with placebo controlled.
Phase I effectively met the primary endpoint, confirming the safety and tolerability of KJ103 in five distinct dose groups with no serious adverse events (SAEs) and infection incidents. In particular, the study did not exclude patients with pre-existing ADA, highlighting the outstanding safety features of KJ103. All adverse events were primarily mild without reaching dose-limiting toxicity (DLT). All dose groups exhibit the ability in cleaving human IgG in dose dependence with consistent PK profiles. For efficacy, KJ103 rapidly and precisely cleaves 95% of IgG within 6 h after administration and maintain a low IgG level (30%) for 1 week. IgG had gradually recovered to baseline one to two months after administration. Furthermore, supplementary exploratory endpoints, including the assessment of ADA levels, were also conducted. ADA levels peaked on day 14 after dosing and gradually decreased after 63 days (42.3% of subjects returned to baseline). ADA levels will also be monitored on Day 120 and Day 180. It might be possible to perform re-dosing of KJ103 after 6 months.

Prior non-clinical studies demonstrated the potential of KJ103 in eliminating the neutralizing effects associated with AAV-based gene therapy. In mice passively immunized with IVIg (intravenous immunoglobulin), KJ103 administration decreased Nabs and enabled efficient gene transfer of AAV9-luciferase. Since Nabs are present in IVIg, the animals treat with IVIg showed no transgene expression. IVIg/KJ103 treated mice showed similar luciferase expression levels compared to control cohort without IVIg treated. KJ103 treatment reversed IVIg inhibitory effect on luciferase gene transfer by degrading Nabs.

AAV-based gene therapy has been a common treatment approach, Nabs to AAV vectors are highly prevalent in humans, represent a limitation to gene therapy. Among the clinical and non-clinical studies, KJ103 has the potential in becoming a pre-treatment of AAV-based gene therapy to overcome the limitation of Nabs to improve efficacy, expand the patient pool, and enable re-dosing of gene therapy. KJ103 is considered a viable solution to address the unmet medical needs of AAV-based gene therapy.

P108

A game-changing RNA delivery platform combining high cell entry and multiple RNA transfer for next generation therapy : LentiFlash®

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Gene & cell therapy approaches show that there is no universal delivery tool for all therapeutic 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(no risk of genotoxicity) to treat or prevent a large variety of diseases. RNA-based therapeutics target specific applications in which a transient expression is required to trigger a cellular process, to modify a genetic sequence or to commit cells into a specific pathway. Once engineered, cells become the effective medicine. Depending on the target disease, RNA must be designed to fit with the therapeutic approach: geneediting, regenerative medicine, or immuno-oncology. The design of the transferred RNA must also be optimized according to the cells targeted, the need for an ex vivo or in vivo approach, gene(s) of interest, expression level, and duration. A game-changing RNA carrier called LentiFlash® has been developed to overcome the current limitations of AAVs and lentiviral vectors such as long-term DNA presence or genomic integration, and necessary repeated administration for multiple genes expression which bring safety implications. LentiFlash® is a bacteriophage-lentivirus chimera capable of delivering biological RNAs that combines the RNA encapsidation system of bacteriophages and the structure of lentiviral particles. Thanks to this new encapsidation method, multiple RNA species can be packaged into a unique LentiFlash® particle. Hence, RNAs are protected from degradation, they are delivered very efficiently into the cell cytoplasm thanks to the large tropism of VSV-G pseudotyping, and they are directly available to be rapidly translated into proteins without any risk of integration into the host genome. LentiFlash® can be used to deliver CRISPR-Cas9 machinery in a single step in Human primary and stem cells, providing efficient gene editing without affecting cell viability, nor stem cell differentiation capacity, and with no genotoxicity. n a regenerative therapy approach, LentiFlash® is used to express two different mRNAs to restore the lymphatic function in the arm of patients with secondary lymphedema (LD). Results show that codelivery of 2 mRNAs candidates abolished lymphedema and restored the lymphatic flow in the limb of mouse model. These LentiFlash® particles will be used as drug product for a Phase I/II clinical trial called Theralymph that will be started next year. Finally, we show the potential of LentiFlash® in immuno-oncology by delivering tumoral antigens into primary murine dendritic cells. These cells are able to present antigens in vivo to the immune system which in turn can efficiently block tumor development in wild type mice. This method gives the opportunity for multiple antigens expression to enhance immune responses, avoiding tumor relapse. These properties, and the ability to produce LentiFlash® using a production platform already compliant with cGMPs, provide a very promising method for safe and efficient therapy in Humans. It offers additional safety considerations compared to other therapeutic approaches.

P109

A novel approach for in-depth analysis of adenovirus and adeno-associated virus capsid proteins using UHPLC–MS/MS

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In-depth characterization of the capsid viral proteins (VPs), e.g., adenovirus (AdV) and adeno-associated virus (AAVs), is immediately needed to ensure consistency in gene therapy products and processes. The FDA and EMA recommend that gene therapy products be identified to ensure product safety, identity, quality, and purity of the investigational product. Peptide mapping can provide detailed information on these proteins, which is crucial for their identification, development, and optimization of the manufacturing processes. Recently, we have developed a new approach for fast and reproducible VP sample preparation that enables the generation of low-volume trypsin digests for single LC-MS/MS analysis. It involves precipitation of VPs followed by solubilization of the protein pellets in sodium deoxycholate (SDC)/N-dodecyl-β-D-maltoside (DDM) for denaturation and digestion. This newly developed "single-tube" preparation methodology reduces variability and processing time for sample preparation by allowing all preparation steps to be done in the same solution. Direct LC-MS/MS analysis of the digested samples enables identification of all structural proteins of AdV and AAV with high amino acid sequence coverage and quantification of associated PTMs. With these results, it can be concluded that the method has sufficient reliability to provide analytical support for the development of AdV and AAV-based gene therapy products (GTPs). Additionally, we anticipate that this approach will motivate further study into monitoring and controlling the host cell proteins of GTPs produced using various vectors and manufacturing processes.

P110

Plasmid engineering and its effects on ITR stability and rAAV production

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Plasmid systems for the production of recombinant adeno-associated viruses (rAAV) have been developed many years ago and are since often used without much attention. In our work, we realized that seemingly small sequence changes can significantly influence and improve rAAV yields. Further, the inverted terminal repeat (ITR) sequences are known for their notorious instability during their propagation in *E. coli* and an inspection of all 123 sequence-verified plasmids available from Addgene as of Jan 2023 revealed that all contained an ITR with a deletion, which is also common for commercial plasmids. Cloning and testing various serotype 2 wild-type like ITR versions uncovered a length dependent improvement of rAAV production at the cost of their genetic stability. Using a low resolution nanopore sequencing approach, we identified strategies to mitigate ITR instability. Last, we created a miniHelper plasmid, which worked well in a traditional three plasmid setting but required redosing when used in combination with optimized RepCap and ITR-plasmids. In summary, small changes to old plasmids unrelated to capsid type and gene of interest can improve rAAV production more than tenfold.

P111

Differentiated transcriptomic signatures detectable in primary human hepatocytes transduced by AAV-based vectors. A potential enabler for *in vitro* safety profiling

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Following treatments with AAV-based gene therapies, multifactorial dose-related toxicities have emerged in clinical trials and have raised concerns with regulators and health care providers. Reported adverse events are not limited to specific vectors, although liver toxicities are prominent and have led to discontinuation of some clinical trials or serious events caused by already approved products. However, the exact molecular pathologies leading to elevated liver enzymes, serious liver injury, and liver failure remain largely elusive. Clinical safety issues are poorly predicted by testing in preclinical species due to differences in biodistribution, transduction efficiencies, payload expression/clearance, and immunogenicity among others. Based on predictive Drug-Induced-Liver-Injury transcriptomics, we set out to build a defined in-vitro platform applicable to RNA sequencing of transduced human liver cells, with the aspiration to add molecular insights to vector safety assessments, aided and informed by transcriptomics profiling. Human liver cell lines (HepG2, HepaRG) and primary human hepatocytes (PHH) were seeded in 384-well plate formats and transduced by AAV2, AAV6, AAV8 and AAV9 based vectors expressing a gfp or luc reporter transgene (N= 8-16/ treatment). Control treatments included buffer controls and treatment with empty capsid (N=8-32/treatment). Time-course studies (up to 4 days) and vector titrations (MOI) were used to optimize transduction efficiencies and cell viabilities assessed by quantitative imaging and ATP measurements. All vectors transduced human liver cells, albeit with varying efficiencies (AAV6>AAV2>>AAV8>AAV9) and PHH were selected for transcriptomic profiling. Unbiased high throughput RNA-Sequencing (htRNA-Seq) was done using ScreenSeq platform, quantifying expression levels for about 10,000 genes in a sample. Differential expression and Gene Ontology (GO) enrichment analysis (e.g., assessing stress response and effect on other intrinsic pathways) was done using a ML/AI supported bioinformatics platform (PanHunter). htRNA-Seq enabled reliable phenotype-independent quantification of transgene expression, with sensitivity similar to or higher than sensitivity of e.g., fluorescence-based quantification. More importantly, htRNA-Seq allowed for unbiased analysis of transcriptomic response to transduction with a correlation between MOI and strength of transcriptomic response. A clear separation of response into transgene-independent and transgene-specific components was identifiable in PHH transduced by AAV expressing reporter genes. Enriched GO terms for transgene independent response components were identified in AAV transduced PHH. In summary, this preliminary study demonstrates that in AAV-transduced PHH quantification of transcriptomic changes provides insight into differentiated responses to AAV vectors and their payloads that can be decomposed in transgene independent and transgene specific components. Further studies will aim to expand this approach into liver micro-tissues and cocultures of mixed cell types with a special view on ML modelling to predict safety liabilities.

P112

Analytical characterisation of an AAV9 gene therapy

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The characterisation of Critical Quality Attributes (CQAs) of recombinant adeno-associated virus (rAAV) is required to assess quantity, safety, purity and potency of a therapeutic product. Cell and Gene Therapy Catapult (CGTC) has previously developed a suite of analytical assays for AAV2 characterisation and subsequently adapted them to characterise the scAAV9 gene therapy product designed by University of Sheffield (UoS): AAV9 ELISA to identify and quantify total titre, ITR2 quantitative PCR (qPCR) and digital droplet PCR (ddPCR) to quantify AAV genomic titre, and a Single-Stranded DNA Virus Sequencing (SSV-Seq) assay to assess DNA impurity levels. All CGTC analytical assays were successfully adapted to AAV9 and they were deemed fit for purpose for the characterisation of UoS current scAAV9 gene therapy product and any other AAV9 therapies they might develop in the future. These assays were also successfully transferred to UoS following a Technology Transfer (TT) process, building up their analytical capability to characterise the AAV therapies they will be manufacturing at their new Gene Therapy Innovation and Manufacturing Centre (GTIMC), one of three UK gene therapy innovation hubs funded by LifeArc and the Medical Research Council (MRC), with support from the Biotechnology and Biological Sciences Research Council (BBSRC), which aims to help advance the clinical development of new genetic treatments, with potential to transform care for millions of patients including those with rare and life-threatening genetic diseases.

P113

Assessing tissue-specific gene therapies in a pre-clinical mouse model of lipodystrophy

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Congenital generalised lipodystrophy (CGL) is a rare and life-threatening disorder, characterized by inadequate adipose tissue development. This leads to the development of severe metabolic complications including hepatic steatosis, lipoatrophic diabetes and cardiovascular disease. There is currently no cure for CGL, and treatment options remain limited. Adipose tissue has emerged as a target for adeno-associated virus (AAV) vectors. We recently revealed that systemic AAV-mediated gene therapy restores adipose tissue development and metabolic health in a pre-clinical mouse model of CGL. We have subsequently investigated whether tissue specific AAV vectors can provide a more targeted form of therapeutic intervention for CGL. We combined AAV8 vectors with the mini/aP2 or thyroxine-binding globulin promoter to specifically target adipose tissue and liver, respectively (titre: 1 x 10^12). Notably, the AAV-mini/aP2 vectors were designed to incorporate a liver-specific microRNA-122 target sequence, to minimize off target

effects and transgene expression in the liver. Our data indicate that systemic delivery of adipose and liver specific AAV vectors restores adipose tissue development and improves metabolic health in mice with CGL. Serum analysis revealed the restored adipose tissue is functional in both liver and adipose specific mice.Curiously, based on previous research, Liver specific gene therapy was not expected to restore adipose tissue development. We are further examining and will present the similarities and difference in adipogenesis between mice given mini/aP2 or TBG AAV therapy. The assessment involves histology, Immunohistochemistry, and quantitative PCR analysis. Additionally, Western blot analysis of the adipose tissue will be utilized to detect transgene expression and potential off-target effects, providing valuable insights into optimizing viral injection dosage.

P114

Identifying cell-type specific AAV vectors for precision lung gene editing

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The advancement of human lung gene editing requires the development of cell-type specific gene editing vectors to improve efficacy and ensure safety. Adeno-associated virus (AAV) has emerged as a clinically relevant biological vector capable of delivering gene editing machinery to targeted cell types. However, its inherently broad tropism across serotypes often leads to unintended and potentially harmful edits in off-target cells and tissues. To mitigate this issue, a novel pipeline combining single cell transcriptomics and a machine learning-assisted directed evolution approach was developed to identify cell-type specific AAV variants. Our workflow involves training machine learning (ML) models to predict AAV viability and cell-type specificity using data generated from two *in vitro* mutant library screens. The first mutant library pool systematically samples the manufacturable sequence space of an AAV capsid which has a combinatorial amino acid insertion within viral protein 1. Sequence data from this library screen in HEK 293T cells trains a ML model to exhaustively predict AAV viability across the entire variant sequence space. Subsequently, a second mutant AAV library which samples the predicted “viable” sequence space is synthesized, screened in porcine precision cut lung slices (PCLSs) – a generalizable lung model – and dissociated into a single cell suspension for scRNA-seq analysis. A second ML model trains on this sequencing data to predict AAV variant enrichment in each lung cell type. By identifying the cross-section of multiple objectives, such as high specificity in alveolar type II cells and low specificity in alveolar macrophages, we can select vectors tailored to fit different lung gene editing experiments. Our findings are experimentally validated by selecting the top five predicted variants with the highest performance for both alveolar type II cells and alveolar macrophages. These selected variants are manufactured, and their cell specificity is assessed in PCLSs. The generation of this AAV sequence-to-function repository is a key building block in our data-driven approach to improving precision lung gene editing.

P115

Riboswitch-controlled IL-12 gene therapy reduces hepatocellular cancer in mice

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Hepatocellular carcinoma (HCC) and solid cancer with liver metastasis are indications with high unmet medical need. Interleukin-12 (IL-12) is a pro-inflammatory cytokine with substantial antitumor properties, but its therapeutic potential has not been realized due to severe toxicity. Here, we show that orthotopic liver tumors in mice can be treated by targeting hepatocytes via systemic delivery of adeno-associated virus vectors carrying the IL-12 gene (AAV-IL12). Controlled cytokine production was achieved in vivo by using the tetracycline-inducible K19 riboswitch. AAV mediated expression of IL12 led to Stat4 phosphorylation, interferon-gamma production, infiltration of T cells and, ultimately, to tumor regression. By detailed analyses of efficacy and tolerability in healthy and tumor-bearing animals we could define a safe and efficacious vector dose. As a potential clinical candidate, we also generated AAV carrying the human IL-12 gene. In mice, bioactive human IL12 was expressed in a vector dose-dependent manner and could be induced by tetracycline, suggesting the combination of tissue-specific AAV vectors with riboswitch-controlled expression of highly potent proinflammatory cytokines could play a role for vector-based cancer immunotherapy.

P116

fDISCO evaluation of AAV-mediated gene expression upon different routes of administration

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Detection methods to assess gene transfer-mediated expression of reporter proteins in tissue requires one to dissect or slice the sample, during which the three-dimensional (3D) structure is lost. On the other hand, live imaging often does not provide sufficient resolution to identify reporter protein expression on a cellular level. Here, we have tested whether fDISCO (3D imaging of solvent-cleared organs with superior fluorescence-preserving capability) could overcome these limitations. To this end, DBA/1 mice were injected intraarticularly (IA), intramuscularly (IM) or intravenously (IV) with AAV-encoding fluorochrome mGreenLantern (mGL). Hindlimbs and livers were isolated three weeks after administration, followed by fDISCO tissue clearing. Cleared tissues were imaged by a light-sheet fluorescence microscope, followed by image stitching to create the 3D structure of the sample. The fluorescent signal of mGL was detectable at the cellular level in the tissue. The highest number of mGL expressing cells were found in the injected muscles (gastrocnemius or quadriceps) of IM groups. The IA group showed visible mGL expression in muscles around joint. The IV group showed the lowest number of mGL expressing cells in the

muscles throughout the entire hindlimb. All routes of administration resulted in mGL expression in liver correlating with the total vector dose injected per animal. In conclusion, fDISCO is able to capture the complete histological information of fluorescent reporter signal, thus being a suitable method to study the AAV-mediated reporter expression on a cellular level in 3D tissue samples.

P117

Modification of AAV9 capsid at different positions affects differently to AAV infectivity in culture

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Attaching various moieties to the capsid of adeno-associated virus (AAV) is a strategic approach aimed at generating novel variants of the virus with enhanced characteristics and capabilities. The capsid of AAV plays a crucial role in determining the virus's ability to transduce target cells and evade immune responses. By modifying the capsid with different moieties, we can alter its properties and potentially improve its performance for specific applications.

In our study, we have utilized a technology that enables the expansion of the genetic code to attach a fluorescent probe, Cy5.5, at four different positions on the AAV capsid. Through the substitution of the original capsid residue with a reactive non-natural amino acid, specifically azide-lysine, we have successfully generated four mutant variants without negatively impacting their production.

By attaching the Cy5.5 molecule to positions M471, A591, Q592, and T593 at varying proportions, we evaluated the infectivity of these modified AAV variants in cultured cells. Our findings indicate that both the position and degree of capsid modification significantly influence AAV transduction. We observed an increase in the number of AAV genomes with higher degrees of modification; however, there was no direct correlation with transgene expression.

Interestingly, we noted a negative impact on transgene expression when the AAV capsid was highly modified. This phenomenon was accompanied by an accumulation of viral particles in the cytoplasm when the capsid contained a higher proportion of Cy5.5 attached to any of the four residues. Therefore, it is important to consider both the degree of modification and the specific position of the attached molecules on the AAV9 surface to potentially modify particle properties without compromising their infectivity.

These findings highlight the significance of carefully controlling the modification of the AAV capsid to achieve optimal transduction efficiency and minimize undesired effects on transgene expression.

P118

Enhanced expression of the human *Survival motor neuron 1* gene from a sequence-optimised cDNA transgene *in vitro* and *in vivo*

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Current adeno-associated virus (AAV) vector therapies rely on the use of very high doses to achieve therapeutic efficiency. This is due to both limited targeting of the relevant tissues after systemic administration and poor transgene expression efficiency of the vector construct. Very significant efforts are being devoted to engineering AAV capsids to improve tissue targeting, but little work seems to be ongoing to enhance transgene expression efficiency. In this abstract we show that significant gains can be achieved by engineering the cDNA transgene to maximise expression efficiency, with positive results both in cell culture and *in vivo*. Spinal muscular atrophy (SMA) is a systemic disease with particular impact on the neuromuscular system. It is caused by mutations or deletion of the *Survival motor neuron* (*SMN*) *1* gene. Three marketed drugs are effective against this disease, relying on either gene addition with an AAV vector of serotype 9 (AAV9) to replace the faulty *SMN1* gene (Zolgensma), or splice-switching of the closely related *SMN2* gene transcript with antisense technology (Spinraza) or a small molecule (Evrysdi). We have developed a novel, sequence-optimised *hSMN1* transgene and produced integration-proficient and integration-deficient lentiviral vectors to initially test it in cell culture. These vectors were driven by cytomegalovirus (CMV), human synapsin (hSYN) or human phosphoglycerate kinase (hPGK) promoters to determine the optimal expression cassette configuration. Integrating, CMV-driven and sequence-optimised *hSMN1* lentiviral vectors resulted in the highest production of functional SMN protein *in vitro*. Integration-deficient lentiviral vectors also led to significant expression of the optimised transgene and are expected to be safer than integrating vectors. Lentiviral delivery in culture led to activation of the DNA damage response, in particular elevating levels of phosphorylated ataxia telangiectasia mutated (pATM) and gamma-H2AX, but the optimised *hSMN1* transgene showed some protective effects. Neonatal delivery of AAV9 vector encoding the optimised transgene to the $Smn^{2B/-}$ mouse model of SMA resulted in a significant increase of SMN protein levels in liver and spinal cord. This work shows the potential of a novel sequence-optimised *hSMN1* transgene as a strategy to reduce the therapeutic AAV vector dose for SMA.

P119

DRG de-targeting of AAV9 gene therapeutics using miRNA regulatory elements

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Dorsal root ganglion (DRG) toxicity has emerged as a platform-wide concern in AAV-based gene therapy. Highly efficient transduction of DRG neurons by AAV and subsequent transgene expression has been shown to lead to satellite cell proliferation, mononuclear cell infiltration, axonopathy and neuronal degeneration in non-human primates. To address this critical issue, we leverage endogenously expressed micro-RNAs that are highly expressed in DRG tissue. We generated multiple AAV9-eGFP expression vectors with micro-RNA binding sites and tested if this strategy can mitigate vector related toxicity in DRG. All vectors were compared to a control AAV9-eGFP expression vector. We have implemented a workflow that efficiently evaluates micro-RNA binding sequences *in vitro* (human iPSCs) and *in vivo* (mouse, NHP).

In vitro, we showed that human iPSC sensory neurons transduced with AAVs suppressed GFP protein only when micro-RNA binding sites were present. We then evaluated these vectors and the effects of these micro-RNA binding sites in mice. Mice were dosed by intracerebroventricular (ICV) injection and were assessed for vector biodistribution, eGFP mRNA, and eGFP protein expression. In DRG, we observed broad biodistribution of all vectors, however GFP mRNA and protein expression were reduced only in mice dosed with micro-RNA binding site inclusion vectors. Multiple additional tissues were also analyzed to assess possible effects of the micro-RNA binding sites on mRNA and protein expression. Following the mouse study, we confirmed the conservation the micro-RNAs sequences in non-human primates. NHP were dosed with vectors via intracisternal magna (ICM) injection. One-month post-treatment, animals were assessed for vector biodistribution, eGFP mRNA, and eGFP protein expression.

Our *in vitro* and *in vivo* data indicate that this strategy can be efficiently used to regulate tissue-specific transgene expression of AAVs, and it supports the inclusion of such elements to mitigate DRG-related safety liabilities.

P120

Unleashing the potential of AI: Solid Biosciences' journey in harnessing AI to overcome vector design challenges

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The field of gene therapy faces several challenges in vector design and manufacturing, resulting in high costs and safety concerns. Vector design has traditionally been a time-consuming and inefficient process of trial and error. Additionally, the safe and cost-effective manufacturing of AAV gene therapies remains a persistent issue in the industry, with empty and partial capsids

contributing to toxicity and immunogenicity concerns. Expensive and inefficient bioreactors further increase the cost of goods sold, impacting the overall affordability of therapies.

Early design decisions often lead to manufacturing challenges that require costly engineering solutions. However, Solid Biosciences has taken a different approach by leveraging long read technologies and Form Bio's AAV QC Workflow to revolutionize their understanding of vector characterization. This provided crucial insights into the problematic aspects of their vectors, such as quality and heterogeneity issues.

Rational design choices were then made using in silico iterations, allowing Solid Biosciences to rapidly evaluate design choices that would have taken months in the wet lab and cost millions of dollars. The incorporation of regulatory cassette elements and bioreactor simulation aided in optimizing vector constructs, while AI algorithms adjusted and tuned the genetic payload and predicted the formation of secondary and tertiary features.

The use of AI models was aimed at the strategic incorporation of novel intellectual property (IP) to outperform competitive designs. Solid Biosciences also deployed AI to focus on novel vector components, evaluate competitive constructs in the literature, and ensure optimization for manufacturing production.

The results of Solid Biosciences' approach so far have been promising. We predict faster time to identify a clinical candidate. Furthermore, it is possible that our clinical candidate requires lowered patient doses during clinical development thereby reducing concerns related to toxicity and immunogenicity. These advancements have the potential to significantly impact the field of gene therapy and improve patient access to effective treatments.

P121

Multiple method determination of AAV integrity for gene therapy

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Recombinant Adeno-associated virus (rAAV) is one of the most used vectors for in vivo gene delivery for the treatment of diseases. It has a great potential to deliver a high efficiency and long-term gene expression.

Nonetheless, the length of the transgene can be an issue for vector design, nucleotides sequence of interest cannot be longer than approximately 4kbp. Moreover, the integration procedure of the vector genome (VG) in the viral capsid is nowadays a large field of investigations.

Depending on the VG sequence, capsid serotype, production and purification process, the full/empty ratio could be impacted.

Some reference techniques are used such as analytical ultra-centrifugation and HPLC-SEC to determine the percentage of full/empty capsids nevertheless the information of partial VG is always not precise or absent (length, sequence, etc.).

Quantitative PCR remains the standard method to quantify VG copies. But recently, droplet digital PCR (ddPCR) was shown as a most robust and reliable method for VG quantification. By ddPCR, a

DNA sequence can be isolate in a droplet to be quantify by one or more targets with different fluorescents probes (or association of fluorescent probes).

We used this technique to isolate an AAV in a droplet, associated to a thermal lysis, and performed a multidimensional ddPCR. Targeting several sequences distributed throughout the VG provides information on its integrity. The presence and absence of some targets along the VG in addition to the VG titer offer a complete, rapid and robust method to characterise a rAAV batch. Integrity results show a heterogeneity in rAAV population with multiple genome configuration: full length genome with all the targets but also different partials genome with many configurations more and less represented.

We also compared two different methods in 2D ddPCR with several dilutions to calculate genome integrity: method developed by Biorad with the linkage and 2D method using concentration of each targeting population. Results show a robust genome integrity calculation with linkage whatever the dilutions performed unlike 2D method.

To complete and compare these data, next generation sequencing methods were used: illumina sequencing and nanopore technology. These two sequencing methods offer the possibility to sequence the VG with by different approaches. Short read sequencing allows to verify the AAV sequence (identity) whereas long read sequencing allow to analyse full-length VG sequence. Genome integrity obtained by ddPCR were compared with this sequencing results.

Incoming studies in these areas for recombinant AAV biology can provide a better understanding of viral associated therapeutic challenges (e.g in rAAVs process optimization, to improve safety and infectivity of batches).

P122

Development of alternatives to Triton X-100 cell lysis for AAV2, AAV5 and AAV8 primary recovery

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Triton X-100 is an effective detergent for recovering biological products from intracellular compartments, but its use is now prohibited by Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) regulations as its degradation product is detrimental to aquatic life. This work aimed to develop alternative methods to Triton X-100 cell lysis for adeno-associated virus (AAV) recovery from HEK293 cells which were REACH-compliant and good manufacturing practice (GMP)-compatible, did not affect product quality and showed comparable product recovery relative to lysis with 0.1% Triton X-100. Two alternative AAV release methods were evaluated for AAV2, AAV5 and AAV8 serotypes: 1) A detergent-free hyperosmotic release method was initially tested at microplate scale using a Design of Experiment approach, before scaling up to 250 mL stirred tank reactor (STR) scale. 2) When not suitable, a cell lysis method using Deviron C16 detergent was assessed in 250 mL STR with concentrations ranging from 0.1 to 0.5%. Finally, successful conditions for either method were scaled up to 10 L STR. For AAV8 serotype, a hyperosmotic AAV release method employed the supplementation of sodium chloride and extended incubation time leading to a 70% VG recovery relative to the lysis with 0.1% Triton X-100. For AAV2 and AAV5 serotypes, a 0.5% Deviron C16 lysis method proved most suitable, resulting in comparable VG recoveries relative to AAV release with 0.1% Triton X-100. No downstream process changes were needed to accommodate these alternative methods.

Furthermore, both methods were scalable to 10 L STR and process related impurities, including residual DNA and host cell proteins, as well as product potency, were not impacted by these methods. In conclusion, this study demonstrates two optimized alternative AAV release methods to substitute the use of Triton X-100 with comparable product recovery and product quality profiles.

P124

Evaluation of new approach for quantification of intact viral genomes without amplification using NanoString technology

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Gene therapy offers new opportunities for the treatment of a range of diseases, from cancer to various genetic disorders. Therapeutic viruses, such as adeno-associated viruses (AAV), are used to deliver genes to target cells. Effective gene therapy requires functional viral vectors with intact genomes; however, current harvesting and purification methods cannot completely separate the mixture of full, empty, and partially filled viral particles. Currently, even the final drug product contains at least some unwanted particles, either with impurities or with fragmented genomes. Currently, several analytical approaches using digital PCR or long-read sequencing are being developed to assess genome fragmentation. However, they require genome amplification as one of the steps in the protocol, which has several drawbacks. Among others, genome amplification can lead to uneven amplification of genome regions and is unable to amplify full-length genomes in one molecule, resulting in genome fragments and consequently affecting the analysis. The sequencing approach on the other hand has some bias against shorter reads, which compromises accurate quantification. We addressed these drawbacks by using a NanoString technology to analyze genome fragmentation of AAVs. This technology was developed for gene expression analysis without an amplification step. Two probes, one biotin-labeled and one fluorescently labeled, are hybridized to the sample (either DNA or RNA), and the complex is then bound to the cartridge, followed by automatic counting of fluorescent probes using specialized equipment. We have developed several probes for the viral genome and have used this approach to assess the integrity of the viral genome. Because even purified viral particles are quite heterogeneous, we first used a plasmid fragment of the viral genome as a surrogate for the viral genome to evaluate the approach for analyzing the integrity of the viral genome. We assumed that the plasmid fragment was intact if all hybridized probes gave similar values. Probes were hybridized as singleplex (one target region per hybridization) or multiplex (multiple target regions per hybridization). Preliminary results from these experiments have shown that counts for genomic regions are not comparable between simplex and multiplex hybridizations. We attribute this to steric interference and inefficient binding of the complex to the cartridge due to multiple biotin binding sites on a single plasmid fragment. We are currently improving the strategy by analyzing different probe combinations and performing experiments using only one biotin and one fluorescent probe. To detect only intact fragments, biotin and fluorescence probes hybridized at opposite ends of the fragment. Nevertheless, we demonstrated the presence of multiple genomic regions without amplification and confirmed that the NanoString approach technology can be used to analyze viral genomes. The accuracy and suitability of this approach for assessing viral genome integrity remains to be confirmed. Such confirmation would have enormous implications for the future of analytical approaches for therapeutic viruses.

P125

3D imaging of whole mice to characterize AAV distribution and transduction using Cryofluorescence Tomography

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Gene therapy discovery requires early characterization of vector distribution and transduction, with factors such as promoters, serotypes, titer, and administration routes having a crucial effect. Careful consideration should be given to these factors when targeting a specific organ or disease indication, to increase on-target and minimize off-target distribution. Current approaches, commonly utilized to characterize AAV transduction, comprise of qPCR, to detect and quantitate vector-genome copies, biochemical homogenate assays, to detect and measure protein expression in organs, or immunohistochemical methods to localize protein expression on a cellular level. Although well-established, these approaches are laborious and require organ selection and isolation, from which a tissue sample is then collected for further analysis. Since these approaches are employed on tissue biopsies/ homogenates they are limited in spatial resolution. A trade off exists between tissue homogenate assays, which allows assaying whole organs but lack spatial resolution, versus tissue biopsies/ section assays, which provide spatial resolution but only sample a small portion of the organ. Taken together, these assays provide an in complete picture of AAV distribution and transduction.

Since many AAV research and development efforts use fluorescent proteins as biomarkers, in vivo fluorescent imaging could also be utilized to determine vector transduction. However, since GFP is the fluorophore used in almost all cases, it introduces many challenges for in vivo fluorescence imaging resulting in low sensitivity and low resolution to properly make reliable conclusions.

We designed an imaging study to characterize and compare AAV7 CAG-EGFP and AAV9 CAG-tdTomato transduction in mice, using cryofluorescence tomography (CFT). A 3D imaging modality that allows for fluorescence imaging in high resolution and high sensitivity across whole intact mice. Reconstructed and co-registered image data demonstrated protein expression across several organs. In some organs transduction was expected, however transduction was also observed in unexpected organs/ regions, that typically don't get sampled for biochemical analysis, these included cranial sinuses, incisors, mandibles, and tongue. Transduction in these organs, and others, was demonstrated by qPCR and IHC, confirming AAV-targeting of previously unexplored regions, as well as validating CFT as an unbiased technology allowing to fully characterize AAV transduction in all organs, thus eliminating unbiased tissue selection. CFT presents a powerful imaging modality, enabling complete visualization of AAV-mediated expression of fluorescent biomarkers and complementing traditional biochemical assays.

P126

Development and optimization of a robust upstream platform for AAV production

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As AAV continues to be a prominent modality in gene therapy, more resources are being invested into AAV manufacturing platforms to allow for cost effective production of these medicines. A wide range of AAV capsid variants, both wild-type and engineered, are deployed to enhance tissue tropism for effective delivery of the corrective gene to maximize therapeutic effect. Although this is beneficial for potency and safety profiles of AAV products, it provides a unique challenge to the process development, as key manufacturing attributes of the AAV differ drastically between serotypes, leading to variable yields and product quality in the bioreactor. The Alexion Genetic Medicines CMC team has invested significant effort to modify the AAV upstream manufacturing process to improve the consistency of the productivity and the quality of AAV produced in the bioreactor. This work includes the development and implementation of the mAAVRx system, a transient transfection platform that has shown high productivity across a range of AAV serotypes. Additionally, transfection and bioreactor conditions were optimized to work synergistically with the mAAVRx system to continue to increase titers to minimize cost of goods. Initial screening work was performed through implementation of DOE based studies in shake flasks, with the optimized results being confirmed in small scale bioreactors. Finally, the upstream process scalability was evaluated, with multiple constructs at the pilot scale and one product being transferred to a 1000L STR as proof of concept for the scalability of the mAAVRx upstream platform.

P127

Optimized AAV *de novo* synthesis platform for efficient AAV packaging to accelerate gene therapy programs

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The adeno-associated virus (AAV) is a powerful vehicle for gene therapy; however, working with AAV vectors can be challenging. Each construct contains two inverted terminal repeat (ITR) sequences that flank the gene of interest. The ITRs form highly stable T-shaped hairpins, which are critical for the replication and encapsidation of viral DNA. These structural features can also make traditional cloning workflows difficult because spontaneous deletions in the ITR region are common and formation of secondary structures makes sequencing challenging. Here we present a novel workflow for gene synthesis and AAV plasmid preparation that maintains ITR integrity during plasmid prep and DNA scale-up. We describe a complete synthetic solution that optimizes AAV viral packaging to maximize successful research results through:

- Rapid identification of mutated or truncated ITR sequences, followed by correction via a synthesis approach

- Proprietary plasmid synthesis and transfection-grade DNA preparation techniques developed to maintain ITR integrity, maximizing AAV packaging efficiency.
- Generation of high-quality viral particles that have high titer and efficacy in transduction to improve gene delivery

The reported research demonstrates a streamlined approach that improves data quality through innovative synthesis and sequencing methodologies. Taken together, this approach allows for a comprehensive and robust solution to enable AAV packaging for gene therapy applications.

P128

Enhancing AAV6 Vector Production for Cell Therapies: Harnessing the Potential of Viralgen's Pro10™ Platform for Scalable Manufacturing

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The field of cell and gene therapy has shown remarkable promise in treating a wide range of diseases and disorders, ranging from genetic disorders to cancer. In order to fully harness the potential of cell and gene therapies, efficient and targeted delivery of therapeutic genes or factors into cells is essential. Typically, the delivery vehicle used in both cell and gene therapies is a viral vector. For cell therapies in particular, lentivirus and retrovirus have been the vectors of choice, and for gene therapies, Adeno-associated virus (AAV) vectors have emerged as the predominantly used tool to deliver genetic material. More recently, however, due to their ability to transduce a variety of cell types, AAV vectors can offer immense potential for advancing the field of cell therapies. More specifically, the serotype AAV6 presents a broad tropism, demonstrated high transduction efficiency in many cell types, enhanced muscle targeting, low pre-existing immunity, and a favorable safety profile, making it an advantageous choice for various cell therapy approaches. For the robust manufacturing of this serotype, Viralgen's Pro10™ production platform has proven reliability and scalability, making it suitable for meeting the demands of large-scale manufacturing for cell therapies. The Pro10™ platform includes a well-established process that promotes the consistent production of high-quality AAV6 vectors, enabling a robust supply to relevant cGMP scales. Viralgen's experience with AAV6 includes the successful manufacturing of 12 batches for toxicological studies and 13 cGMP (250/500 L cell culture) batches, in addition to over one hundred 2L batches, showcasing a commitment to excellence and continuous advancement in the field. Run data collected show consistency and robustness across different batches produced, despite variations in the GOI within the AAV6 constructs tested, confirming the efficacy of using a manufacturing platform for reliable production of AAVs to advance cell therapies.

P129

Centrifuge-free clarification of adeno-associated virus crude cell lysates using a novel alluvial filtration method

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Adeno-associated virus (AAV) vectors have emerged recently as promising DNA delivery tools for *in vivo* gene therapy. However, efficient AAV downstream processing is still challenging because serotypes differ in physicochemical characteristics, making it difficult to establish standardized purification processes. Clarification of AAV is a particularly critical process step. AAV harvest, like for other viruses, often involves cell lysis. This leads to a difficult-to-filter cell lysate and rapid filter clogging. In our experiments, we evaluated the applicability of diatomaceous earth (DE) as a filter aid for clarifying AAV crude lysates. DE filtration was found to be a viable clarification method for AAV2, AAV5, and AAV8. On the basis of a design of experiments approach, the concentration of DE was identified as the most relevant factor influencing the loss of AAV particles. By maintaining DE levels below 0.181 mg DE/10^{10} AAV, AAV loss during DE filtration was limited to <2%. Compared to filtration combined with a prior centrifugation step, the use of DE reduced manual handling time 3-fold and increased filter capacity by a factor of 3.5. In addition, we were able to show that the type of DE had only a negligible effect on the filtration performance. Our results demonstrated that filtration using DE as a filter aid is an efficient method for the clarification of different serotypes of AAV.

P130

How to account for the noise in next-generation sequencing datasets?

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Next-generation sequencing (NGS) has revolutionized genomics research since the early 2000s. Thanks to this technique, some fields of evolutionary biology such as directed evolution and deep mutational scanning have benefited from the availability of big data, improving the characterization of variants with desired properties. In these cases, NGS is used to screen biological libraries in order to measure the concentrations of different mutants by reading and counting them. Yet, because of several amplification steps in the experimental protocols, like PCR amplification or bacterial growth, NGS datasets suffer from large noise errors that are often neglected. To gain deeper insight in this phenomenon we have developed a statistical model of the entire NGS protocol, incorporating the exponential processes, inspired by Galton-Watson process theory. Our model predicts a simple proportionality law between the variance and the mean of the counts data, that we have validated in both real data and in-silico simulations. Finally

as a practical application, we have created a tool that uses this validated noise model to infer and denoise the frequencies of nucleotides or amino-acids occupation.

P131

Cryo-EM structure of AAV8 and epitope mapping of CaptureSelect AAVX

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As the need for novel therapeutic options grows, researchers require innovative strategies and transformative technologies to overcome the limitations of traditional drug discovery methods. Cryo-electron microscopy (cryo-EM) has emerged as a powerful technique that can help accelerate this process. Despite the relatively recent adoption of cryo-EM in discovery efforts, multiple cryo-EM-supported therapies have already entered the clinic. The broad applicability of native state imaging underpins the rapidly increasing usage of cryo-EM in biologics pipelines, which include monoclonal antibodies, cell and gene therapy, and all phases of vaccine development.

Here, we highlight two significant applications of cryo-EM in developing gene therapy delivery vehicles like adeno-associated viruses (AAVs). Firstly, we demonstrate how simple 2D cryo-EM imaging can support continuous quality assurance by characterisation of AAV particles based on a 2D image for shape, size morphology and empty/partial/filled. Secondly, we exemplify the use of 3D cryo-EM structures in capsid engineering. We determined the 2.3 Å resolution cryo-EM structure of AAV8 in complex with single-domain antibody fragment CaptureSelect™ AAVX (Figure 1). This antibody fragment is the ligand in Thermo Fisher's POROS™ CaptureSelect™ AAVX affinity resin*, enabling large-scale AAV purification of a broad range of naturally occurring or synthetic AAV serotypes. Using the structures for epitope mapping of AAVX, we identified which amino acid residues of the capsid are needed for AAVX-mediated purification, thereby informing the rational design and capsid engineering to develop delivery vehicles with improved efficacy and targeting ability.

*For Research Use or Further Manufacturing. Not for diagnostic use or direct administration into humans or animals.

P132

Engineered heart-specific AAV vectors from a novel next-generation platform (next-CAP) with cross-species selection for cardiac-targeted gene therapy

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Hereditary and acquired heart failure (HF) remains a leading cause of death worldwide due to the lack of curative treatment options. By targeting the underlying genetic and molecular origins, gene therapy bears the potential to transform future clinical care in this important healthcare segment, but crucially depends on safe and efficient delivery of the therapeutic gene to cardiac cells. In this regard, engineered adeno-associated viruses (AAVs) selected for enhanced heart tropism are viewed as the best gene transfer vectors by minimizing transduction of off-target organs upon simple systemic administration.

Here, we describe the development of next-generation cardiac-specific AAV capsids based on our experimental-bioinformatic AAV capsid engineering platform (next-CAP) using mouse, farm pig and non-human primate models. By integrating DNA shuffling and peptide display approaches, we generated a highly diverse capsid library with over 100 million variants derived from novel mammalian heart AAV isolates. This library underwent a two-step in vivo evolution procedure in the three animal models, where cardiac-enriched capsid sequences were amplified from heart tissues in the first round to generate the AAV library for the second in vivo screening. Utilizing a unique molecular identifier strategy for each AAV, we combined long- and short-read sequencing to select novel capsids that displayed highest enrichment in heart tissues compared to all other organs, including the liver. Notably, subgroups within the top heart-specific AAV capsids displayed up to 99.5% sequence identity, which highlights the presence of novel conserved heart-targeting motifs. As producibility was another inherent screening criteria, all identified AAV capsids already provide scalable manufacturing characteristics.

Our proprietary collection of novel heart specific AAV capsids now enables us to systematically develop the best therapeutic ensembles of capsids, promoters, and transgenes for further assessment of dose-expression, efficacy, and toxicity to address various HF indications in appropriate model systems. In summary, we established an efficient and versatile platform for engineering next-generation AAV capsids tailored for heart-specific gene therapy of rare and common cardiac diseases that currently lack curative treatments.

P133

Unlocking insights: Exploring genetic profiles of rAAVs through orthogonal DNA profiling methods

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During the production of recombinant AAVs (rAAVs), the vast majority of capsids package the correct, full-length transgene. However, a notable proportion of packaged DNA is undesired, originating from residual plasmid DNA or host cell genomic DNA. Regulatory authorities require not only careful quantification but also a comprehensive characterization of these impurities to ensure a thorough understanding of the safety, immunogenicity and efficacy profile of a rAAV batch. Quantitative polymerase chain reaction (qPCR) and droplet digital polymerase chain reaction (ddPCR) remain the standard methods for DNA quantification of both the vector genome and DNA impurities. Previously, we demonstrated the utility of a multiplexed ddPCR assay to evaluate length information of impurities, such as the kanamycin resistance gene (*nptII*). Nevertheless, this method's reliance on sequence-specific primers reduces its ability to provide comprehensive information about all diverse encapsidated impurities.

Next-generation sequencing (NGS) represents a significant advancement as it circumvents the need for a priori information. Long-read NGS technologies, such as Nanopore and SMRT sequencing, can provide reads of several kilobases originating from a single DNA molecule. Consequently, long-read technologies offer a unique opportunity to provide length and sequence integrity as well as sequence recombination information of encapsidated single-stranded AAV genomes, as well as all packaged impurities derived from the production platform.

To elucidate existing challenges associated with both PCR-based methods and NGS, we have generated data to explore the complementarity of these approaches including also data from the former gold standard for genome integrity analysis: alkaline gel electrophoresis. Our findings indicate that an orthogonal use of these methods allows for the most accurate evaluation of the length profile and transgene integrity of encapsidated DNA. While NGS currently lacks the ability to perform an absolute quantification of DNA impurities, a comprehensive analysis is possible in conjunction with data from ddPCR approaches and alkaline gel electrophoresis. The combined approach offers a more robust and accurate characterization of packaged DNA, encompassing amount, length, integrity, and identity.

P134

Applying Fiber/Knob-modified adenoviral vectors to increase transduction efficiency in endothelial cells for gene therapy

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Helper-dependent adenoviral vectors (HDAdVs) lacking all viral coding sequences are capable of delivering single or multiple transgenes including regulatory elements up to a maximum of 35 kb. HDAdVs are absent of any viral genes, which is in sharp contrast to the frequently implemented first-generation adenoviral vectors (fgAdVs), which lack the early E1 and E3 transcription units. Therefore, HDAdVs display lower toxicity and immunogenicity, resulting in long-term transgene expression in quiescent tissues such as the liver. Nevertheless, there is a significant setback to the most used serotype 5 (Ad5)-based HDAdVs, such as the limited transduction efficiency among some tissues due to the low expression of its primary attachment receptor (coxsackie adenovirus receptor, CAR).

Different techniques were utilized to target AdVs to certain tissues. Examples include genetic modification of fiber, knob, or capsid proteins. In our recent work, we observed that fgAdV based on serotype 17 (Ad17) can achieve enhanced endothelial cell tropism. Here we aimed at transferring this knowledge to HDAdVs by modifying the Ad5-based helper virus to carry the Ad17 fiber/knob.

We first constructed two helper viruses based on serotype 5 (HV5) featuring the Ad17 knob (K17) and in another version the Ad17 fiber (F17). Chimeric helper virus genomes were generated by homologous recombination of the helper virus genome containing plasmid pHV5 derived from the classical helper virus NG163 for HDAdV5 production. Currently these two novel helper viruses are in the rescue process. The helper viruses HV5K17 and -F17 will be used for production HDAdVs containing therapeutic genes. All new vectors will be tested in vitro and in vivo for transgene expression in endothelial cells.

Meanwhile, using our reporter-tagged human adenovirus library we performed high-throughput screening in a panel of human tumor cell lines. We found that, adenovirus types 16 and 50 (Ad16, Ad50) also achieved higher transgene expression levels (up to three-fold) compared to Ad5 in an endothelial cell line (EA.hy926). Furthermore, Ad16, Ad21 and Ad37 displayed enhanced transduction in tumor cell lines derived from osteosarcomas (Saos-2, MG-63 and U-2 OS). Therefore, after the protocol establishment with the HDAdV5F17 and –K17 chimeric vectors, fiber/knob proteins from those newly identified types (Ad16, Ad21, Ad37 and Ad50) will be also transferred to the helper virus system.

Taken together, our new helper virus system (such as HV5F17 and -K17) will enable optimized helper-dependent vectors production, and transduction in low-CAR expressing cells, such as endothelial cells.

P135

Optimization of AAV2 vector-production in scale-down multi-parallel bioreactor systems: multi-parameter process optimization using high-throughput analytical tools

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The adeno-associated virus (AAV) is one of the most promising vectors in gene therapy and currently the most expensive pharmaceutical product in the world. One of the main contributors to the high price of AAV-based drugs is the expensive manufacturing processes involved, necessitating process optimization to enable cost-efficient production. Traditional process optimization approaches are known to be time-consuming, costly, and labor-intensive. In this context, the application of multi-parallel scale-down bioreactor systems proves valuable for identifying critical process parameters and accelerating process development. Furthermore, the integration of high-throughput analytical techniques expedites this optimization process.

Here, we developed a transient production process for AAV2 vectors in scale-down bioreactor systems, utilizing advanced semi-automated analytical techniques, including biolayer interferometry (BLI)-based Octet® platform and the Incucyte® Live-Cell Analysis system. These cutting-edge techniques enabled us to analyze capsid as well as functional titers in a high-throughput manner, significantly reducing the workload for operators. We implemented a systemic approach to evaluate critical process parameters that directly influence the final yield of functional AAV2 vectors. Specifically, we focused on key parameters such as cell densities and plasmid DNA concentration within 15 ml multi-parallel bioreactor systems. Subsequqntly, we scaled up the selected setup from 15 ml to 250 ml multi-parallel bioreactor systems. Within the 250 ml scale, we further investigated the effects of bioprocess mode and temperature variations. Once the optimized setup was determined, we successfully scaled it up to a 1L benchtop bioreactor. The scaled-up bioreactor allowed us to validate the feasibility and reproducibility of our optimized process on a larger scale.

Our findings demonstrate the efficacy of utilizing multi-parameter process optimization in scale-down bioreactor systems, coupled with high-throughput analytical tools, for cost-efficient AAV2 vector production. The implementation of these strategies contributes to the establishment of robust, reproducible, and cost-effective AAV production, ultimately making AAV-based gene therapies more accessible to patients in need.

P136

Exploration of AAV formulation buffer excipients capable of increasing recovery rates and reducing aggregation during buffer exchange with centrifugal concentrators

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The adeno associated virus (AAV) formulation process affects key manufacturing parameters, such as recovery rate, types, and levels of product-related impurities in the final product. The recovery rate, in turn, impacts the cost of goods for a manufacturing run, while formulation-derived impurities, e.g., capsid aggregates, compromise the safety of the final product. Here, we have focused on optimizing the buffer exchange process with centrifugal concentrators by exploring different excipients, with the aim to maximize recovery rates and minimize the formation of aggregates. We started by testing two benchmark buffers with a challenging serotype, AAV2, known to be prone to aggregation during the buffer exchange process. With an AAV2 benchmark formulation buffer, i.e., a DPBS Luxturna®-like buffer, we observed a 19.4% mean recovery rate (n=4, SD=9.86%) with 100 kDa MWCO (Molecular Weight Cut Off) centrifugal concentrators. While with the second benchmark buffer, the patented ELT buffer, composed of a salt (NaCl), osmolytes (histidine, glycine, trehalose), and a surfactant (polysorbate 80), we have observed a 96.4% mean recovery rate (n=2, SD=11.81%), albeit with varying aggregation. This led us to test if the DPBS Luxturna®-like buffer could be optimized by single ELT buffer excipients. No single ELT excipient led to an increase in recovery or monodispersity, suggesting the excipients have a synergistic effect on particle aggregation. Next, we tested if the DPBS Luxturna®-like buffer could be optimized by increasing its ionic strength, since AAV2 aggregation was shown to be prevented above an ionic strength of 200 mM. Therefore, we substituted NaCl with 68.45 mM sodium citrate in the formulation of DPBS Luxturna®-like buffer (final ionic strength of 439 mM and 306 mOsm), but we did not observe a reduction in aggregation or losses. Substituting NaCl with another higher-valency salt (final ionic strength of 850 mM and 306 mOsm) led to <10% aggregation, and a 55.58% mean recovery rate (n=2, SD=15.68%). Subsequent comparison tests with AAV1, AAV2, AAV3B, AAV6 and AAV9 showed aggregation of <10% for all serotypes with the higher-valency salt, while DPBS Luxturna®-like buffer had 26.7% aggregation for AAV2, and <10% for other serotypes. Mean recovery rates for AAV1, AAV2, AAV3 and AAV9 were higher in the high-valency salt (94.3%, 54.7%, 42.5%, 100%, respectively, n=3), compared to DPBS Luxturna®-like buffer (65.7%, 9%, 10.5%, 88.8%, respectively, n=3), with exception to AAV6 (32.4% versus 59.8% in DPBS Luxturna®-like buffer, n=3). Together, our results show that high ionic strength and/or excipients synergistically preventing AAV2 aggregation are key to develop a formulation buffer which maximizes recovery rates and minimizes the formation of impurities during the buffer exchange process with centrifugal concentrators.

P137

Quantification and characterization of helper plasmid residuals in rAAV drug products

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Recombinant Adeno Associated (rAAV) Vector based human gene therapy is an evolving frontier for transformative molecular medicines. rAAV vectors are manufactured using various scalable platform bioprocess technologies. However, the most prominent technology utilizes a triple DNA transfection of suspension HEK293 cells followed by a series of chromatography-based purification steps. The Adenovirus Helper DNA, which is one of the three DNA components in the transfection, may consist of inactive 'Fiber' and 'Hexon' adenovirus type 5 (Ad5) structural protein sequences. Thus, quantification and characterization of the residual fiber and hexon sequences in the final rAAV drug product is critical to attest its safety. The residual fiber and hexon DNA sequences in the drug products were quantified using a droplet digital PCR (ddPCR) assay. To further characterize the size of the residual sequences, the fragmentation was analyzed using a multiplex-ddPCR based linkage analysis. The potential expression from the residual fiber and hexon sequences was analyzed by a cell-based viral vector transduction assay quantifying mRNA using RT-ddPCR and RT-qPCR. In addition to the mRNA quantification, protein expression was analyzed using capillary western blotting. The contamination of the Fiber or Hexon sequences compared to the nominal titer was ≤ 0.01% copies/VG for both Fiber and Hexon. The multiplex ddPCR linkage analysis of residual fiber and hexon residuals demonstrated that most of these sequences are fragmented. No mRNA quantification or protein expression was observed for residual Fiber or Hexon sequences thus displaying that the sequences are incapable of expression upon cellular transduction. Thus, these efforts demonstrate successful development of assays for quantification and characterization of the helper plasmid residuals. An extremely low quantification of Fiber and Hexon sequences which are largely fragmented and incapable of expression was observed thus attesting the safety of the process and the product.

P138

Engineering viral-like particles from human commensal virus as a vector for DNA delivery

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Anelloviruses are circular ss-DNA viruses that constitute the majority of the healthy human virome. They are remarkably diverse, and have been found in various biological samples but with low immunogenicity and no disease association. These features make Anello virus an attractive candidate vector for DNA delivery. Here, we describe our efforts to show that engineered viral-like particles (VLPs) derived Anello virus capsid can be produced in vitro, can be genetically engineered to carry exogenous DNA cargo, and that the exogenous DNA cargo can be expressed in cells transduced with Anello VLPs. We further show its potential application by placing engineered tRNAs and genome editors in the cargo, paving the way to engineer Anelloviruses for delivery of therapeutics.

P139

Novel hepatocyte-specific synthetic enhancer-promoters for liver targeted gene therapies

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Major objectives of viral vector-mediated gene therapy are to deliver the therapeutic transgene and protein product specifically to the target site where it is needed, to limit off-target expression and side effects, and to reduce the viral titre required for therapeutic impact. Rational design of novel synthetic promoters with highly defined transcriptional properties is likely to help to meet these challenges.

The liver is a key target for gene therapy, with an increasing number of liver-targeted gene therapies in clinical trials, and first therapies gaining regulatory approval. We therefore sought to develop novel hepatocyte-specific synthetic promoters with properties suitable for inclusion in viral vectors.

We employed a bioinformatics-assisted approach to mine genomic data from a combination of open-source and in-house transcriptomic and epigenetic datasets to identify cis-acting regulatory motifs. These motifs were synthesised and assembled in a combinatorial manner to generate bar-coded synthetic enhancer-promoter libraries driving expression of a fluorescent reporter in lentiviral or adeno-associated viral vectors. These libraries permitted massively parallel fluorescence-assisted and NGS-quantified activity screens in hepatoma or hepatocyte cell types. The screening campaign identified a large panel of synthetic enhancer-promoters with a >10-fold range of activity in hepatoma cell lines (Huh7 and HepG2), and iPSC-derived and primary human hepatocytes. Moreover, for many of these enhancer-promoter combinations activity was restricted to the desired hepatocyte target cells and was absent or very limited in non-hepatic control cell types. Thus, we demonstrate that novel synthetic enhancer-promoter combinations identified using our promoter discovery platform provide strong and hepatocyte-specific expression that is on par with, or exceeds that, of other commonly used promoters to drive transgene expression in hepatocytes. Our results highlight the potential utility of synthetic enhancer-promoters in driving transgene expression, and we further demonstrate that viral vector context can influence the performance of champion enhancer-promoter sequences.

P140

Insights into AAV promoter activity and helper virus components interactions

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Recombinant Adeno-associated viruses (AAVs) have emerged as highly promising vectors for gene therapy due to their favourable safety profile, ability to mediate long-term transgene expression, and broad tissue tropism. Efficient production of rAAV vectors is essential for their successful translation into clinical applications. Traditional methods involving transient transfection or stable AAV producer cell lines through helper virus infection pose limitations in terms of scalability and

biosafety. To address these issues, the development of stable AAV producer cell lines that are devoid of replication-competent virus has garnered significant attention. This approach offers improved biosafety and the potential for scalable, consistent, and cost-effective production of rAAV vectors. However, current strategies have not yet achieved titers comparable to those obtained in transient transfection- based production. We believe that this discrepancy stems from an incomplete understanding of the intricate interactions between AAV and helper virus components. The aim of this study is to further elucidate these interactions and pave the way for their manipulation.

In this study, we focused on investigating the independent activity of AAV promoters. We constructed several plasmids containing AAV2 promoters (p5, p19, and p40) driving the expression of the reporter gene Gaussia luciferase (*Gluc*). These constructs were transiently transfected into mammalian cell lines (293, A549, and Vero) in the absence or presence of a pHelper cassette harbouring adenoviral helper factors (E2a, E4, and VARNA). The strength of the AAV promoters was assessed by comparing the Gluc expression to a mock control. Among the tested cell lines, AAV promoters p5 and p40 exhibited the highest activity, while promoter p19 consistently displayed the weakest activity. Furthermore, co-transfection of the pHelper cassette resulted in an increase in AAV promoter activity in 293 cells. However, in non E1-coding region cell lines (Vero and A549), the presence of E2, E4, and VARNA alone was insufficient to enhance AAV promoter activity. We are currently investigating AAV promoter conformations with inducible potential.

Additionally, we aimed to determine which components of the pHelper cassette are essential for AAV production. We generated seven plasmids with various deletions and combinations of different components in the adenoviral genes E2a and E4. Using transient transfection, we produced rAAV5, rAAV8, and rAAV9 vectors. Through this approach, we identified the adenoviral genes that significantly impacted vector genome (VG) productivity and the ratio of empty to full particles. Notably, the effect was less pronounced for the rAAV5 serotype.

These findings contribute to our understanding of AAV production and the role of specific components in promoter activity and VG titer. The knowledge gained from this study will ultimately facilitate the development of improved strategies for large-scale production of high-titer, high-quality rAAV vectors, thereby advancing the field of gene therapy.

P141

AAV-based liver-targeted gene therapy for MNGIE: proposal for a clinical trial

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Mitochondrial NeuroGastroIntestinal Encephalomyopathy (MNGIE) is an autosomal recessive disease caused by loss-of-function mutations in *TYMP*, the gene encoding the enzyme thymidine phosphorylase (TP). In MNGIE patients, TP deficiency leads to systemic accumulation of the nucleosides thymidine (dThd) and deoxyuridine (dUrd). This results in an imbalanced pool of mitochondrial deoxyribonucleoside triphosphates (dNTPs), causing mtDNA depletion/deletions and the introduction of somatic point mutations, eventually leading to a defect of oxidative phosphorylation and the clinical phenotype.

MNGIE is an ultra-rare fatal neurodegenerative condition, with an estimated incidence of ~1/million births, and average symptom-onset in late teens. The most debilitating symptom is severe gastrointestinal dysmotility and cachexia, leading to a median age of death ~35years. Current treatment options are allogeneic hematopoietic stem-cell (HSCT) or liver transplantation (LT). Restoring normal TP activity in blood or liver cells reduces circulating levels of dThd/dUrd. Over time, this arrests clinical progression, and reverses some of the symptoms and clinical features. However, these procedures are limited by the difficulty of finding compatible donors and are associated with significant risks and mortality in affected individuals who often have multiple MNGIE-related co-morbidities.

We have previously obtained evidence that administration of the *TYMP* coding region using a liver-targeted AAV2/8-vector is effective at reducing dThd/dUrd levels and preventing some neurological and motor dysfunctional features in the mouse model of MNGIE. We aim to bring this gene-therapy strategy to patients through pre-clinical toxicity/biodistribution studies, and a Phase I/II clinical trial to test safety and biochemical efficacy (dThd/dUrd levels).

Based on a successful preliminary proposal, we have recently been invited by LifeArc to submit a full funding application for their Gene Therapy Innovation Fund. We have now partnered with the UKRI/LifeArc Gene Therapy Innovation and Manufacturing Centre (GTIMC) (University of Sheffield, UK) to develop manufacturing plans, conduct pre-clinical toxicity and biodistribution studies, and obtain approval and funding for a clinical trial. We are currently preparing regulatory applications, planning a patient and public involvement exercise, and surveying clinicians to estimate the number of MNGIE patients that could potentially be recruited across Europe.

P142

Computational modeling-guided directed evolution for the discovery of novel AAVS with blood-brain barrier penetration capability

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Adeno-associated viruses (AAVs) have emerged as common gene delivery vehicles for treating numerous human diseases. Genetic therapy using AAV in neurological disorders is a leading platform because they are considered safe, low immunogenic, and long-term persistency. However, effectively enabling the noninvasive transportation of AAVs across the blood-brain barrier (BBB) poses a significant challenge in terms of efficient clinical application. In this study, we employed an integrated approach combining experimental selection strategies and computational modeling to synthesize chimeric capsid encoding AAVs and predict their ability to traverse the BBB through AAV capsid-receptor binding. To discover the AAV capsid candidates with improved tissue- or cell-specific tropism for brain targeting, we generated a library of engineered AAV capsids using directed evolution techniques, including AAV capsid DNA shuffling and peptide display. To evaluate the binding affinity between the generated AAV candidates and the brain receptors, we employed a comprehensive methodology that integrated AI-based structure prediction models with molecular modeling techniques. The outcomes of this investigation have the potential to guide the application of noninvasive, customized gene therapy for brain disorders.

P143

Comparative analysis of neDNA™3 and plasmid DNA for recombinant Adeno-Associated Virus (rAAV) Production

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neDNA™3 is an enzymatic DNA technology produced by rolling circle amplification and end ligation that can be used for rAAV production or other DNA-intensive bioprocesses. neDNA™3 has numerous advantages over plasmid DNA (pDNA), including rapid production, simplified scalability, and minimal residual kanamycin resistance or other bacterial sequences. While neDNA™3 has been demonstrated to perform well in rAAV production, there are limited data directly comparing optimized processes. In this work we assessed the productivity, packaging, and infectivity of rAAV generated from neDNA™3 and pDNA in both AAV8 and AAV2 serotypes. Using identical DNA sequences (Transgene, Ad Helper, RepCap) and Pro10™ production cells, pDNA or neDNA™3 processes were optimized for individual ratios and total DNA per cell. These optimized processes were then scaled into 3-L bioreactors to compare the viral productivity, packaging efficiency, and infectivity. Importantly, we found that pDNA and neDNA™3 had nearly identical process optima. Capsid and viral genome titers suggested that neDNA™3 provided equivalent or better total capsid and viral genomes for both AAV8 and AAV2 when compared to pDNA. 260/280 absorbance ratios of affinity-purified virus confirmed the equivalent packaging efficiency between neDNA™3 and pDNA. Together, our results demonstrated that neDNA™3 produced rAAV with titers equivalent to, or greater than, those generated with pDNA whilst simultaneously improving the speed and scalability of DNA manufacturing, with limited contaminants.

P144

Nuclear proteome analysis of adeno-associated virus producing HEK-293 cells

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Recent efforts to improve and upscale recombinant adeno-associated virus (rAAV) production are hampered by insufficient knowledge of the cellular determinants of rAAV formation. In this study, we focused on the nuclear proteome of adherent HEK-293 cells during rAAV production. For this proteomic approach, we generated three types of samples each in triplicate: i) not-transfected HEK-293 cells (HEK-sample), ii) HEK-293 cells for rAAV production transfected with a triple plasmid system comprising a helper plasmid, a RepCap plasmid of serotype 2, and a gene of interest (GOI) bearing ITR plasmid (AAV-sample), and iii) HEK-293 cells transfected with the ITR-GOI plasmid and a noncoding plasmid (MOCK-sample). Cells were harvested 24 h and 72 h post transfection (ptf). Nuclear extracts were generated by fractionation and protein samples were prepared for mass spectrometry (MS) measurements by filter aided sample preparation (FASP). Peptide masses were acquired with a nanoLC-Orbitrap-MS system. By label-free quantification protein abundance changes were evaluated. In total, 3384 proteins in all samples were identified with at least one unique peptide. Protein data were filtered for significantly regulated proteins by a minimum 2-fold regulation and a p-value of 0.05. To elucidate relevant cellular events due to the

transfection procedure, we compared the AAV-samples and MOCK-samples with the HEK-samples at each time point. In the AAV-samples 24 h and 72 h ptf, 235 and 318 proteins were up- and 347 and 212 proteins were down-regulated, respectively. In the MOCK-samples 24 h and 72 h ptf, 215 and 132 proteins were up- and 425 and 116 proteins were down-regulated, respectively. First, we took a closer look at proteins regulated in the AAV-samples, which partially were also regulated in MOCK samples. In this set, eight up-regulated proteins are known to belong to the viral host defence system (identified by STRING queries), some with a connection to the host defence of adeno and adeno-associated viruses. Hence, these proteins are interesting targets to improve rAAV production yields. Finally, we focused on proteins that were only regulated in rAAV producing cells at 24 h and 72 h ptf and filtered for a Benjamin Hochberg corrected p-value of 0.05 with at least five identified peptides. This analysis resulted in five up-regulated and six down-regulated proteins. The fact that they are regulated 24 h and 72 h ptf implies that they may influence the whole rAAV production period. Because of the strict analysis parameters, these 11 proteins are interesting targets for further studies.

P145

Quantitation of AAV ratios in a dual-vector system using SV-AUC

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Hearing loss at birth is termed congenital hearing loss and affects approximately 1.7 out of every 1,000 children born in the United States. Hearing loss can significantly impact mental health, cognition and language development. The majority of permanent, congenital hearing loss cases are sensorineural and result from a single gene defect. Decibel Therapeutics is a clinical-stage biotechnology company dedicated to discovering and developing transformative treatments for hearing loss, one of the largest areas of unmet need in medicine.

Dual vector AAV gene therapies pose manufacturing challenges where formulation of the drug product (DP) necessitates controlling drug substance (DS) concentrations to target a specific ratio of the two vectors and final total vector concentration. Control of vector ratios relies on accurate titer measurements. Since each vector uses an identical serotype and capsid, and the transgene mass only differs by a few kDa, ddPCR or qPCR are currently the only methods available which can distinguish the vectors in the final DP. Quantitative PCR methods are known to have significant assay variability and an orthogonal biophysical method based on first principles would be beneficial in testing and evaluation of dual-AAV drug products.

Sedimentation velocity analytical ultracentrifugation (SV-AUC) has become the “gold standard” for characterization of the empty, partial and full capsids of gene therapy products (i.e. AAV and Adenovirus). Other techniques such SEC-MALS, Mass Photometry and CDMS (Charge Distribution Mass Spectrometry) also claim to parse out the capsids, however, the resolution of these techniques is severely lacking. In this body of work, SV-AUC was implemented in a dual AAV vector system where the difference in GOIs was ~400 nucleotides. The SV-AUC experiments were optimized to accurately measure the percentage amount and the ratio between the two AAVs with less than 10% error as compared to ddPCR. The results of this work highlight the resolution and accuracy of the capsid quantitation by SV-AUC.

P146

Plasmid Engineering Considerations in Upstream Manufacturing of Adeno-Associated Virus

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Permanent hearing loss of all severities is reported in 1.7 per 1000. Congenital hearing loss is associated with subpar cognitive development, language development, academic achievement, and reading. Severe hearing losses are associated with poorer outcomes. More than half of all congenital hearing loss are caused by single gene mutations while biallelic mutations can cause severe to profound deafness. Decibel Therapeutics is focused on developing AAV gene therapy products to address the unmet therapeutic need for monogenic hearing loss. AAV manufacturing processes are typically low productivity (titer) occurring from both upstream production and downstream yield. These low productivities pose a significant challenge meeting material requirements for clinical supply, release testing, and stability. Typically, increased material needs involve scaling-up the process which is inefficient and costly. New approaches to increase AAV vector production without increasing the overall batch size are needed. Mammalian systems for AAV production often utilize a triple transfection method where three plasmids are co-transfected into suspension or adherent HEK production cells. One plasmid contains the gene of interest (GOI) for the disease indication. One plasmid contains the AAV rep and cap genes to support DNA replication, packaging and capsid formation of the AAV. The final plasmid contains helper genes from adenovirus. Previous studies have demonstrated that re-engineering plasmids can help improve upstream yield and productivity, therefore, an AAV1 rep/cap plasmid was developed to help improve these attributes. The Rep2 and AAV1 Capsid genes were unaltered between the control and the engineered plasmids. Multiple plasmid backbone iterations were screened to evaluate the effect on AAV production. One rep/cap construct was identified that resulted in a log increase in titer, when compared to the control. This increase was seen in both capsid (cp/mL) and genome (vg/mL) titers. Analytical analysis via intact mass (mass spec) confirmed that the capsid proteins produced with the two rep/cap plasmids have equivalent mass and peptide sequence. Ultimately this titer increase has allowed for the production scale to decrease from an 800L batch size to a 50L batch size. Further studies are ongoing to understand how the changes in plasmid design were able to increase productivities allowing for a further optimized process.

P147

In silico modelling of the behavior of intrinsically disordered peptides to facilitate AAV vector design

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Peptide design is the key component for enhancing the targeting capability of adeno-associated virus (AAV)-based gene therapy. Identification of promising peptides requires intensive computational modeling to explore the expansive peptide sequence space.

Intrinsically disordered peptides (IDP) are short and flexible peptides involved in numerous biological functions. Due to their flexibility, classical experimental methods cannot fully explore

the conformational space of these peptides. Their interactions with proteins are determined by their internal flexibility and are affected by the environment, rendering *in silico* protein-peptide complex prediction more challenging. Despite major advances in protein structure predictions, the performance of AlphaFold2 and other similar approaches to systematic prediction of IDP structure or protein-peptide complexes are still limited. Specifically, these approaches are not trained on short peptides and do not explore alternative binding modes for protein-peptide complexes.

Exploring alternative binding modes of protein-peptide complexes starting from an IDP sequence using molecular dynamics (MD) simulations paired with artificial intelligence (AI) for structure prediction could provide promising results. In this work we focus on analysis of the behavior of peptides in a solvation environment using MD, with the aim of extracting relevant conformations suitable for molecular modelling.

A framework was developed comprising four parts: (i) generation of 3D conformations from IDP sequences, (ii) classical MD simulation, (iii) unsupervised identification of the most relevant physical-based descriptors and (iv) clustering of the MD trajectory. The outcome of this framework will be used for molecular modelling tasks (e.g., docking, scoring, AI).

This internal framework allowed us to transform the 3D molecular structure of peptides into a representative list of numerical descriptors of the physico-chemical behavior of the peptides. These peptides were then clustered using unsupervised machine learning algorithms to capture conformational states and discard transition states in MD trajectories.

A systematic evaluation of our approach was performed on multiple protein-peptide complexes from the PepBDB database.

P148

Overcoming downstream processing challenges to enable industrialization of adeno-associated viruses for gene-therapy

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Today, commercially approved Adeno-associated virus (AAV) gene therapy product such as Hemgenix®, Roctavian® and Zolgensma® are administered in doses >1e15 vg. These high doses bring quality and safety concerns to reach regulatory acceptance criteria for residual DNA in the bulk drug substance which should be below 10 ng/dose. Another challenge is to reach a high ratio of full versus empty particles while maintaining a high recovery. In this work, the downstream process development of an AAV platform for serotypes AAV2 and 8 is described which will overcome these major challenges. To enhance residual DNA removal and to accelerate the time required for process development, a design of experiment (DoE) methodology was applied to determine the most efficient endonuclease type in combination with the step-location, the incubation temperature, incubation time and harvest composition as most important factors. It was demonstrated that a DNA log removal of >1 was achieved compared to a reference process. DNA was further removed by several chromatography steps. The recovery of full AAV particles was shown to be highly dependent on the obtained quality in the bioreactor harvest. Several strategies were explored to further separate full and empty particles while maintaining a high recovery! In conclusion, advancements in AAV downstream process development have

significantly contributed to improving DNA removal and full-empty separation to ensure safer and more efficient AAV based therapies.

P149

Generation of novel AAV serotype with enhanced infectivity, specificity, and lower toxicity via π-Icosa™ AAV Capsid engineering platform

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Adeno-associated virus (AAV) is commonly used in gene therapy research but has limitations for targeted gene therapy. *In vivo* gene delivery faces challenges like ineffective uptake, off-target infections, and neutralization by pre-existing antibodies. AAV capsid engineering is a promising solution to overcome these challenges.

PackGene's π-Icosa™ system is an advanced AAV capsid engineering platform that constructs and screens capsid libraries to identify top variants with enhanced infectivity and reduced off-target effects. The process involves generating a diverse capsid gene library using deep mutation and rational design, with the assistance of an AI-based prediction model, followed by in vivo testing to verify biodistribution and tissue tropism. This data guides subsequent library design and testing to identify the most efficient AAV variant.

The π-Icosa™ system has successfully engineered AAV variants with improved specificity in the central nervous system (CNS), muscle, and other tissues. A novel serotype, AAV-PG008, was created by inserting random peptides into the AAV9 capsid, resulting in better CNS targeting compared to known serotypes. Another variant, AAV-PG007, was generated through a combination of rational design and directed evolution, showing increased muscle targeting and reduced off-target effects in mice and monkeys. Another set of AAV6 variants generated by π-Icosa™ system show 10 times higher infection efficiency to primary human T-cells than the wildtype.

PackGene's π-Icosa™ AAV Capsid Engineering Platform provides a reliable solution to the generation of novel AAV capsid with improved infectivity, specificity, and reduced toxicity, enabling the development of more precise and effective gene therapies.

P150

Development of a robust potency assay: the gateway to understanding the structure-function relationship in AAV gene therapy

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One of the essential components of successful drug development is product characterisation. Determining the critical quality attributes and the potential impact on efficacy helps to ensure consistency of product lots used in the clinic. The methods used to measure product potency should be robust, stability-indicating, and reflective of the mechanism of action. This presentation demonstrates an approach to develop a robust *in-vitro* method for evaluation of functional potency with minimal assay variability for AAV gene therapy. Additionally, it includes findings from a forced degradation study, using thermal stress to establish the structure and function relationship using a comprehensive suite of analytical assays. Thereby, it emphasises the appropriate use of a functional potency assay to indicate changes in the stability of an AAV gene therapy candidate. This presentation will also highlight the AAV gene therapy candidate life cycle, by drawing a parallel between other potency assays that show the *in-vitro* activity of the AAV candidate at various stages of infection. Lastly, it attempts to provide an explanation for the observed loss in AAV drug stability and potency.

P151

A rapid and agile approach to AAV manufacturing

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Adeno-associated viruses (AAVs) have gained considerable attention as promising tools for gene therapy due to their ability to efficiently deliver therapeutic genes to target cells. To translate AAV-based therapies from research laboratories to large-scale manufacturing processes, several challenges need to be addressed.

One of the primary challenges in AAV manufacture, lies in scaling up the production of AAV vectors, as research laboratories commonly produce vectors using a serum containing adherent cell culture processes which are not amenable to large-scale manufacturing.

Transitioning to suspension cell culture systems is essential for achieving higher production yields, but it necessitates the development of robust and scalable processes which can be costly and time consuming to achieve as each unit operation is optimised and standardised across the platform.

To de-risk this process, we propose an agile approach to process development, which significantly shortens process development time and costs, but still leads to a greater than 10-fold increase of viral productivity at harvest and demonstrates the ability to double the number of full capsids post anion exchange chromatography.

Using the principles of quality by design (QbD) we identified 2 key stages that are vital to both viral productivity and overall product quality; transfection, and anion-exchange chromatography (AIEX).

A statistical design of experiments (DoE) approach was applied to determine the relationship between key factors of transfection in order to define our optimal transfection space. Initially a face-centred, three-factor central composite design is used to simultaneously assess and optimise the viral genome (VG) at harvest. Using this approach, the total DNA concentration, the cell density, and the volume of transfection reagent are methodically assessed to determine the impact of each variable across this system. Following the improvement in harvest titre, an evaluation of the purification process was undertaken.

To initiate the AIEX optimisation, an initial linear gradient was run with the intention to identify elution profile and provide insight on the required conductivity to elute the different populations. As linear gradients can be difficult to execute at large scale, an isocratic elution is usually preferred to increase process robustness.

With the information gathered from the previous linear gradient elution, a step elution was pursued, with initial formulations for wash and elution steps identified. Subsequently, a DoE approach based on a central composite design was undertaken to determine the relationship between critical formulation parameters and their effects on VG recovery and percentage full particles.

To confirm the robustness and suitability of this approach for manufacture, we investigated the impact of the changes when scaled within our stirred-tank reactor (STR) platform using the Ambr® 250 (Sartorius), then further scaled to 40 L in a BioBlu 50C Eppendorf with purification completed.

This study showcases a robust and rapid strategy that paves the way towards efficient, economical, and scalable AAV manufacturing that is suitable for all stages of product development.

P152

Insertion of membrane protein-specific nanobodies into AAV9 improves the transduction of glioblastoma cells

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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) are non-enveloped and 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 the VP1 capsid protein led to markedly enhanced transduction of target cells expressing the corresponding membrane protein. We now report application of this technology to improve the transduction of glioblastoma cells with AAV vectors. Glioblastoma brain tumors are lethal and highly therapy-resistant with limited success of immunotherapies. Thus, novel therapeutic strategies are needed. Human as well as mouse

glioblastoma cells overexpress the GPI-anchored ecto-nucleotidase CD73, a purinergic enzyme that contributes to the generation of immuno-suppressive adenosine and a cold tumor microenvironment. We equipped the AAV serotype 9 as vector of choice for the CNS with a CD73-specific nanobody by genetically inserting the nanobody into the GH2/GH3 loop of VP1. AAV9 displaying the CD73-specific nanobody greatly enhanced the transduction of several human and mouse glioblastoma cells *in vitro*. This paves the way for using nanobody-displaying AAV to express immune-activating cytokines and other effectors in the gliobastoma microenvironment in order to turn a cold into a hot tumor microenvironment.

P153

A machine learning model to design manufacturable AAV peptide insertion capsid libraries

Adeno-associated virus (AAV) capsids play a crucial role in gene therapy. However, identifying sequences which are easily manufactured remains a challenge. In this study, we present a machine learning model to predict the manufacturability of AAV capsid sequences, using a viability metric as a proxy for ease of manufacture.

Our model was trained on a publicly available dataset of production outcomes. Using a deep learning approach, we trained the model using 100,000 data points from the annotated dataset. The model exhibited excellent performance on a hold-out test set, with a Spearman's rank correlation of 0.90 between the model score and the measured viability score. On a separate AAV9 peptide insertion dataset, the model correctly identified 80% of capsids which were not produced.

Next, we experimentally validated our model using an AAV9 peptide insertion library where we inserted 10-mer peptides into loop VIII . We used the model to rank a set of 440 million randomly generated peptides and selected the 5583 highest-ranked peptides for the experiment. We found that 99.8% of the model-generated capsids had a higher viability score than WT, with average enrichment values five times as great as the AAV9 WT capsid.

Our machine learning model offers an efficient and reliable tool for predicting the production viability of AAV capsid sequences. We believe by streamlining the selection process, our model accelerates the development of AAV-based gene therapies, facilitating the translation of promising candidates into clinical applications.

P154

Advancing AAV: Novel sequencing solutions for quality control in gene therapy

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Interest in gene therapy-based disease prevention and treatment has grown rapidly over the last decade, however there are still hurdles to overcome and progress to be made in the field. Recombinant adeno-associated viruses (rAAV) have become the vector of choice for virus-mediated gene therapy due to their non-replicating nature, high-titers, low immunogenicity, and low genotoxicity. 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. Next-generation sequencing (NGS) offers 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 AAV genome and inverted terminal repeat (ITR) regions, with detection of potential mutations, truncations, and contaminants. Both platforms require conversion of the single-stranded genome to double-stranded DNA prior to library preparation; however, high-fidelity protocols for this step are lacking. Additionally, an efficient bioinformatics pipeline is needed to generate interpretable results from massive amounts of NGS data.
Here we describe our novel proprietary workflows, starting with plasmid sequence confirmation, including ITRs. Depending on these results, ITR correction or new synthesis of AAV plasmid options are available. Following packaging, QC results using both NGS platforms with supported from a regulatory-compliant Sanger assay. Sanger ITR sequencing and sequence correction upstream alleviates potential downstream issues in viral packaging. The combined NGS approach 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 AAV manufacturing in advance of FDA or EMA filings.

P155

A differentiated end-to-end process suspension-based platform for the manufacturing of AAVs

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Recombinant adeno-associated virus (rAAV) is among the most widely used viral vectors for gene therapy. However, vector manufacturing remains a challenge as the demand is far greater than the available capacity, hence manufacturers tend to scale up the process which impacts the costs as well as the quality of the AAV produced. Additionally, manufacturing processes are variable from

one AAV serotype to another, increasing the time for process development, scale-up and having AAV ready for clinical supply.

We have developed a robust suspension platform process based on our proprietary HEK293 cell line, that minimises batch variability and builds quality into the product by design. This process decreases complexity, increases the flexibility for scale-up, and thereby decreases the cost of goods (COGs).

Our platform is an end-to-end process solution that is differentiated from the industry. This process platform was optimized for yield at best possible quality, to align with the rigorous regulatory constraints that are continuously evolving while allowing for flexibility and adaption to specific customer program needs. This platform is fully scalable and proven for a number of capsid serotypes, and vector sizes and we are continuously expanding our data to show universal applicability over a wide range of AAV products.

We present here an overview of our platform starting from vial thaw to vial fill, outlining the unit operations that are differentiated from industry. In addition, we show data for the scalability of this platform as well as productivity and quality. Our process platform significantly expedites the timeline to generate clinical grade AAVs to support our clients with their clinical programs.

We further innovate AAV CMC to support the field in bringing AAV gene therapy to more common diseases with high patient numbers and high regulatory bars. Our commitment is to develop the safest possible products for our clients and their patients.

P156

Production of high AAV titers with increased full capsid fraction in a high cell density, serum-free suspension process

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Adeno-associated viruses (AAV) are the vector of choice for a wide range of gene therapies, with a key focus on rare diseases but a growing shifting towards higher prevalence indications. This trend, combined with the dose requirements sometimes exceeding 10E15 vg, means that manufacturing capacity will increasingly become a bottleneck for the clinical progress of these life-saving therapies. Industry has begn to address this challenge by developing upstream platforms with suspension-adapted cell lines in order to benefit from the large capacity offered by stirred tank bioreactors (STRs). However, scale-up in STRs forces developers to compromise on product titers and quality to reach high capacity due to the non-linear nature, and therefore is more complex, costly, and time-consuming, which can cause delays in the time to market. The field is thus in dire need of alternative technologies that can address the above-mentioned challenges to produce cost-efficient, high-quality gene therapy products at scale.

In this session, the speaker will present a comprehensive study performed using an intensified platform for AAV manufacturing using a suspension-adapted HEK293 cell line in a serum-free media. A high-performance production process will be highlighted, including the following key results:

- Achievement of >50 million viable cells/mL
- A 3-fold increase in cell-specific productivity
- A 5- and 10-fold lower concentration of host cell protein and host cell DNA in the collected product, respectively
- A 3-fold increase in full/empty capsid ratio

Building on the results of the study, the key benefits of this intensified manufacturing platform will be highlighted, including drastic reduction in the processing volume, operating footprint and resulting cost of goods for AAV manufacturing. Taken together, the data presented in this session will thus support a credible alternative so STR for large-scale, cost-efficient production of high-quality AAV vectors for gene therapy.

P157

Effect of WPRE sequence on circular RNA expression from AAV vectors

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Circular RNAs are an RNA species believed to act mainly as non-coding RNAs regulating cellular networks. They are generated by spliceosomal splicing in a process called backsplicing, where the splicing donor of a downstream exon splices nonlinearly into the acceptor of an upstream exon. The regulatory effects on cell function have mainly been proposed to be through interactions with RNA binding proteins and other non-coding RNAs such as micro RNAs and long non-coding RNAs, though some circular RNAs have also demonstrated translation potential. This capability for controlling cellular signalling networks makes circular RNAs attractive novel therapeutic targets, suggesting that some of them could be utilised in gene therapy for example via overexpression from a viral vector. Understanding how to optimise the backsplicing process resulting in circular RNA formation in a vector context is then crucial for harnessing this therapeutic potential. Here, we have studied the effect of woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and its placement on circular RNA expression from adeno-associated virus vectors. We demonstrate that WPRE enhances reporter expression both inside and outside the backsplicing sequence without directly enhancing backsplicing efficiency. We also investigated the effects of introns and studied the subcellular localisation of the various constructs. These findings offer insights into optimising circular RNA vector design and more broadly support the conclusions that inclusion of WPRE might enhance expression from viral vectors by more than one independent mechanism.

P158

A two-stage design of experiment approach to develop a scalable and cost-effective AAV production platform

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Recombinant Adeno-associated Virus (rAAV) vectors are a leading modality in the treatment of monogenic diseases, further aided with the development organ specific serotypes, which has not only improved both the safety and efficacy of the therapies but created new therapeutic targets including in the Kidney. However, to realise the demand of these new clinical indications new and innovate strategies are required to increase rAAV productivity within manufacture. A critical step in the manufacture of AAV therapeutics is transient transfection, a complex workflow relying upon expensive plasmids and chemical transfection reagents that are suitable for use within a Good Manufacturing Practice environment, (GMP). Successful transfection relies on the interaction of multiple factors, included but not limited to; cell number, plasmid ratio, DNA concentration, transfection reagent to DNA ratio which are required to assemble and generate the vector. Given the numerous interactions, a one factor at a time approach would be a time consuming and expensive method to assess the interactions that contribute to vector production. To help address this challenge, we sought to develop a systematic approach to optimise AAV production via a two-stage design of experiment (DoE) strategy at a 30 mL scale, to define our optimal transfection space using shake flasks. Initially a face-centred, three-factor central composite design is used to simultaneously assess and optimise the viral genome (VG) at harvest, via the assessment of key transfection parameters. Using this approach, the total DNA concentration, the cell density and the volume of transfection reagent are methodically assessed to determine the impact of each variable across this system. Subsequently, the optimal condition from the first DoE, based on the highest viral genomic titre was carried forward with the aim to maintain high VG titre but enrich the number of full capsids at harvest. To achieve this a secondary design of experiment methodology, a mixture design, was applied. Improvements in full capsids, post transfection was confirmed using a high-performance liquid chromatography (HPLC) method of quantification. Whilst shake flasks are a good investigation tool to screen many conditions, this format lacks both the scalability and control that bioreactors can provide, with stirred tank reactors able to maintain and tightly control a user defined biological environment for viral production. To confirm the robustness and scalability of this proof of concept we investigated the impact of the changes when scaled within our stirred-tank reactor (STR) platform using the Ambr® 250 (Sartorius), a fully automated and scalable multi-parallel mini bioreactors system then later scaled up to 2L, Biostat® B-DCU (Sartorius). This study showcases a robust strategy that paves the way towards efficient, economical, and scalable AAV manufacturing.

P159

Development and technology transfer of scalable platform for transient AAV production

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Adeno-associated viral (AAV) vectors have been established as safe and effective delivery vehicles for gene therapies. However, current methods for AAV manufacturing are not optimal for the production needed for gene therapy applications. High cost of goods, low process yield, and poor product characterization are all metrics that require substantial development and improvement within this emerging field. Currently, most AAV vectors are manufactured using an adherent process, and the demand currently exceeds capacity. While switching from a classical 2D approach to a suspension process using single-use bioreactors might be appealing to meet the requirement in terms of doses and patient number for the late stage of development, this can come at the expense of laborious and costly comparability studies. Hence, a reliable, low-risk manufacturing platform at the desired scale should be identified early during development. Therefore, there is a clear need to develop the next generation of upstream and downstream processes using a suspension cell line in stirred-tanked single-use bioreactors. The Cell and Gene Therapy Catapult has established a platform process to produce an AAV vector utilizing transient transfection of a suspension-adapted HEK293 cell line, with the intention of transfer to recipients, for scale-up and utilization within commercial manufacturing in a GMP environment. Here, we present an end-to-end scalable suspension platform for AAV manufacturing at 2 L scale and the associated technology transfer workflow.

P160

Optimisation of formulation parameters affecting AAV stability

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A number of promising adeno-associated virus (AAV)-based gene therapy (GT) products have recently reached patients, however, the formulation landscape for AAVs remains bland. Transport and storage at ultra-low temperature (<-65oC) is often required to mitigate potency and genome loss, aggregation, and adsorption - challenges associated with the poor stability of AAVs in current formulations. We investigated formulation parameters such as buffer type, salt and sugar type and concentration and pH that affect the physicochemical and biological performance of representative AAV serotypes. We found there was not always agreement between physicochemical stability and potency when changing formulation parameters. Selection of an analytical panel and definition of stability indicating quality attributes (QAs) is therefore critical during early AAV formulation screening. We further conclude that a ‘sweet spot’ needs to be identified between a sufficiently potent formulation and optimal physicochemical stability, which

would facilitate the handling, reduce the costs and, ultimately make GT available to all patients in need wherever they are based.

P161

Scalable recombinant adeno-associated virus (rAAV) production in HEK293 cells with histidine-rich peptide as transfection reagent

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Manufacturing of rAAV-based viral vectors in the plasmid/HEK293 system is performed by co-transfecting plasmids encoding the vector genome (Vg) and the proteins required for viral vector production. The transfection reagent (TR) used for delivering the plasmid DNA to the host cells should meet several criteria to be successfully implemented in large scale production. In addition to high efficiency, consistency, scalability, availability of cGMP grade in required quantities and at an acceptable cost, the optimal stability of DNA:TR complexes and a low complexation volume are particularly important parameters for scaling up the production. Currently, most of the commercially available transfection reagents lack one or more of these desirable attributes.

Histidine rich peptides are known for their ability to deliver nucleic acids and proteins to target cells. However, to our best knowledge, their utilization for viral vector production has not been reported. Here, we highlight the benefits of using a histidine rich peptide (HRP) as a transfection reagent in rAAV production process in HEK293 cells. The key advantages include high transfection efficiency, the industry leading rAAV yield exceeding 1e12 Vg/mL, and scalability of the transfection process facilitated by high stability of DNA:HRP complexes and a low complexation volume.

P162

A novel AAV vector production medium for large scale manufacturing

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Recombinant adeno-associated virus vectors (rAAV) is widely used in gene therapy because of their ability to deliver genes into both proliferating and non-proliferating cells, and sustain long-term target gene expression in non-dividing cells. rAAVs are produced in HEK293 lineage cells expressing adenovirus-derived E1A and E1B. For large scale manufacturing, suspension culture is more suitable than adherent culture because it allows for mass production. In this

regard, there is a demand for optimal culture medium specialized for AAV production in suspension culture of HEK293 cells.

We have developed a novel high-yield rAAV vector production medium for suspension culture of HEK293 cells. This medium is chemically defined, serum-free, protein-free and animal origin-free. To evaluate our novel AAV production medium, 3 different commercially available HEK293 based suspension cell lines were cultured to compare the growth capacity and viability in shaking flask with other commercially available medium. The viable cell densities in our medium reached more than 1×10^7 cells/mL and the cell viability were more than 80% after 7 days culture. Our novel media showed the equal or better performance to other medium in terms of cell proliferation.

rAAV has many serotypes, and it is known that the host range and viral characteristics differ depending on the serotype. So rAAV productivities of various AAV serotypes（AAV1, AAV2, AAV6, AAV8）were compared with a commercially available medium. The rAAVs were produced by transient transfection using AAVpro® Helper Free System (Takara Bio), and 3 days after transfection rAAV were extracted by AAV Extraction Solution (AAVpro® Cell & Sup. Purification Kit Maxi,Takara Bio). The viral genomic copy numbers were measured by the quantitative PCR using AAVpro® Titration Kit (for Real Time PCR) Ver.2 (Takara Bio). As a result, the rAAV yields produced in our medium were equal to or higher than a commercially available medium.

Moreover, using our novel medium we have produced rAAV2 in a 2.4 L bioreactor system and harvested rAAVs 3 days after transfection. The titer of AAV2 was reached to 3×10^13 vg/L, and the cell viability after transfection was maintained at a high level at over 90%.

These results show that our novel medium can be optimal and useful for large scale manufacturing of rAAV in suspension culture system.

P163

Comparison of analytical technologies for the assessment of full/empty capsid *ratio* of AAV viral vectors

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Recombinant Adeno-Associated vectors (rAAV) are among the most used viral delivery systems for *in vivo* gene transfer due to their high efficiency and good safety in a broad variety of cell types and therapeutic indications. The standard for rAAV production is to obtain one specific nucleic acid sequence packaged in one icosahedral AAV capsid composed of VP1, VP2, VP3 viral proteins. Nevertheless, the current methods of rAAV production and purification still result in a heterogeneous vector population, in particular with the presence of undesired empty particles or intermediate particles filled with incomplete DNA sequence. The determination of full/empty capsid ratio is a critical quality control requirement for rAAV production. Multiple analytical methods are currently being used. Analytical ultracentrifugation sedimentation velocity (AUC-SV) is considered as the gold analytical technology allowing the measurement of relative amounts of each particle subpopulation in the rAAV vector batch, in addition to particle aggregates, based on the sedimentation coefficients of the different components. However, AUC method has its

limitations including the sample volume needed for the analysis, the limit of detection and the cost of the specific equipment needed. Today, a large panel of other technologies for the assessment of full/empty rAAV *ratio* are being used or evaluated such as mass photometry, spectrophotometry, size exchange chromatography or capillary electrophoresis, but all present their own set of limitations.

We have evaluated different rAAV batches produced from different manufacturing platforms by different methods assessing full/empty capsid *ratio* to compare and correlate the data. We will present the pros and cons for the different technologies tested and discuss their potential to release quality control testing in routine usage.

P164

Tetracycline Enabled Self-Silencing Adenovirus (TESSA™) Pro system enables highly efficient propagation of adeno-associated virus vectors

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Adeno-associated virus (rAAV) based vectors are attracting enormous interest for gene therapy, however, efficient and scalable production of rAAV to meet preclinical and clinical demands remain challenging. We recently described a new self-silencing helper adenovirus system entitled 'Tetracycline-Enabled Self-Silencing Adenovirus' (TESSA™) for the delivery of AAV rep/cap genes and the AAV transfer genome, using two TESSA™ vectors, for efficient rAAV manufacture without adenovirus contamination.

To expand the utility of the TESSA™ vectors, we explored the process of rAAV manufacture by propagation in HEK293 cells using TESSA™ expressing AAV Rep and Capsid of interest. This unique process, entitled TESSA™ AAV Propagation (TESSA™ Pro), removes the need to encode the rAAV transfer genome within a second TESSA™ vector and allow existing rAAV material to be propagated without transfection. By co-infection of HEK293 cells using rAAV vector stock and TESSA™ RepCap, the TESSA™ Pro enabled amplification of >5,000-fold of input rAAV vector material, yielding up to 4E+11 – 1E+12 vector genome copies (GC) per mL of cell culture. The TESSA™ Pro is particularly efficient for propagation of AAV serotypes that exhibit a high-transduction efficiency in HEK293 cells, including AAV2 and AAV6, which allows high rAAV productivity from a relatively low rAAV vector input of 50 GC per cell. In addition, to enable efficient manufacture of AAV serotypes that are less permissive at infection of HEK293 cells, we further developed a capsid-exchange approach, wherein the rAAV genome of interest is packaged in the AAV2/6 capsid to allow efficient infection of HEK293 alongside a TESSA™ vector encoding AAV rep and an alternative capsid.

Importantly, we observed that the TESSA™ Pro method is highly efficient at rAAV genome packaging and is capable of generating up to 90% full capsids before purification. Presumably, the single-stranded rAAV genomes are rapidly replicated and packaged after nuclear entry, and by passing the requirement for rescue of the AAV ITR embedded within the double-stranded DNA structure that is associated with all other rAAV manufacture systems to date. Additionally, the TESSA™ Pro method has the additional benefit of reduced reverse packaging of DNA sequence contaminants that flank the AAV ITRs, which raises the intriguing possibility that the quality of

rAAV preparations may be improved by propagation. TESSA™ Pro provides a flexible approach to rapidly manufacture high quality rAAV vectors for both pre-clinical and clinical research pipelines.

P165

Screening of transfection reagents using a Design of Experiment approach

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Maximizing productivity and percentage of full capsids is a constant challenge for AAV manufacturing in order to treat patients with the safest and most efficient product possible, and also to reduce the cost of goods for a better affordability. Improving these aspects requires collaborative work from USP, DSP and analytical teams.

At Genethon, the current AAV manufacturing process consists in a transient tri-transfection of suspension HEK293 cells. Among the parameters that influence strongly the productivity and the percentage of full capsids are the cell line, the transfection reagent, and the design of the plasmids. To determine the optimal combination for productivity and percentage of full capsids, a DoE approach has been carried out to screen 7 transfection reagents on 3 cell lines at shake flask scale. In a first attempt, the predictions given by the model after analysis did not perfectly fit with the reality of biology, as some of the optimum conditions proposed could not be reached. New data need to be generated to consolidate the model and provide higher knowledge. However, this approach allowed to select new combinations that could surpass the standard, which were integrated into a new DoE. The results of both sets of experiments will be presented.

P166

Production of low cross-packaging AAV capsid libraries in insect cells

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Expediting directed evolutionary studies aimed at selecting Adeno-associated virus (AAV) capsids with novel properties depends on the production of high-quality libraries of sufficient depth and titer with low levels of cross-packaging. So far, the platform of choice for producing these capsid libraries is based on HEK293 cells. HEK-derived AAV capsid libraries exhibit good titer and depth properties, but are prone to cross-packaging due to the requirement of transfecting high amounts of DNA. We developed an alternative method of AAV capsid library production in insect cells and compared it against the HEK library production platform. Variation was introduced in our proprietary scaffold and subsequently amplified via a three-step process: 1. Insertion of a barcoded library into the VR-VIII region of the AAV5 capsid gene via Gibson assembly; 2. Library synthetic upscaling via rolling circle amplification; 3. Generation of covalently closed fragments via TelN digestion. To produce the AAV library, a low amount (<0.1pg/cell) of library was transfected

into expresSF+ cells, followed by an infection with a baculovirus expressing Replicase. Qualitative analysis of our insect cell library revealed that our method yielded libraries of high titer, while sufficient complexity was carried over throughout the process (>25%). Furthermore, analyzing cross-packaging introduced by our method and comparing it against libraries produced on the HEK platform revealed a significant improvement in favor of our insect cell produced library. Taken together, we anticipate that our novel method of AAV library generation in insect cells will generate libraries of superior quality suitable for accelerated directed evolutionary studies.

P167

Potency assay cell line development for ocular gene therapy vectors

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When producing good manufacturing practice (GMP) adeno-associated virus (AAV) drug products for clinical or commercial use, the ability to assess their potency is an essential part of release and stability. Cell-based potency assays are a regulatory requirement for commercialisation of AAV gene therapies but poor AAV *in vitro* transducibility as well as inability to test expression due to the use of tissue-specific promoters in the viral vector hinders the development of efficient and robust assays. Similarly, basic pre-clinical research, where easy-to-implement tests for faster paced experiments and iterations are desirable, suffers from the same limitations. We therefore set out to examine ways with which to increase vector transducibility as well as establishing ocular promoter trans-activation in the most commonly used cell lines (HEK293 and HeLa). We assessed different methods to increase the transducibility of AAV5 and AAV8 vectors as well as trans-activate photoreceptor-specific (Rhodopsin kinase) and RPE-specific (RPE65) promoters. In dose-ranging experiments, we identified potent dCas9-mediated trans-activators for both promoters, and established exogenous factor supplementation that increased the transducibility of both capsids multiple folds over baseline. Further studies will enable us to combine these in an *in vitro* cell-based potency assay platform for GMP batch release and stability testing.

P168

Comparing AAV2 and AAV9 tropism and transgene expression in mouse tissues after intravitreal and subretinal injection

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The anatomical features of an eye make it an excellent target for gene therapy. The eye is easy to access and systemic biodistribution and side effects are minimized because of the tight barriers in the eye. Gene therapy relies on efficient gene delivery and transgene expression in the target cells. This study aimed to compare the biodistribution and transgene expression after intravitreal (IVT) and subretinal injection of AAV2 and AAV9 vectors expressing enhanced green fluorescent

protein (EGFP) and AAV9-empty vector. Viral vector biodistribution in the mouse eye and off-target tissues were determined by qPCR. GFP expression and GFP transduction efficacy was verified from cryosectioned tissue samples and by using immunohistostaining for GFP. As a result, viral vector copies were found in the eyes after all AAV2- and AAV9-EGFP injections. In addition, vector copies were found in distal off-target tissues such as the liver after both injection groups of AAV9-EGFP. In the eye AAV2-EGFP IVT injection transduced mainly the ganglion cell layer (GCL) whereas subretinal injection transduced mainly the retinal pigment epithelium (RPE). Both injection groups of AAV9-EGFP efficiently transduced the retina thoroughly. In off-target tissues GFP expression was detected in the liver especially after both injection groups of AAV9-EGFP. GFP expression was also found in small parts of the brain after all injection groups except after AAV9-empty injections. Overall there were more GFP-positive samples in the AAV9 injection groups compared to the AAV2 injection groups. In addition, electroretinography (ERG) was done to assess the effect of vectors on retinal function.

P169

Innovative nanoparticles for *ex vivo* and *in vivo* genome editing

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The field of genome editing for gene therapy has experienced an unprecedented boost these last years through the discovery and design of new genome editors. However, to deliver these new systems *ex vivo* to primary cells, or *in vivo* directly into patients, there are several interlink challenges to address: suitable biosafety, efficacy and stability. Existing vector systems do not combine these characteristics. For example, LNPs have generally a good level of biosafety but their use are often limited by their efficacy and stability. Conversely, the vectors derived from viruses have a good efficacy and stability, but usually a low level of biosafety due to their DNA forms. Furthermore, AAV which is mostly used for in gene therapy is sorely limited by its cargo capacity, affecting its efficacy to deliver genome editors which are large.

GEG Tech has designed new generation of nanoparticles combining the advantages of LNPs and viral vectors. These new generations are constituted by a viral packaging enabling a good efficacy and stability, containing non-viral RNA and/or protein conferring a high level of biosafety. We have demonstrated that these new nanoparticles are very efficient to entry in primary cells and are able to induce genome editing *in vitro* and *in vivo* in the retina of rd12 mice to restore *rpe65* expression thanks to a prime editing system efficiently deliver by our nanoparticles.

P170

A high-throughput potency assay accelerates the optimization of process parameters for rAAV production

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Transient transfection of HEK293 cells remains the current gold standard to generate recombinant adeno-associated viral vectors (rAAV) for clinical applications. The assessment of therapeutic activities of rAAV, often indicated as potency, is required by regulatory bodies to demonstrate clinical relevance because potency is normally linked to the biological properties and the claimed mechanism of action. This characteristic makes potency one of the most important attributes of rAAV. According to FDA's recommendation, the assay should incorporate measurement of gene transfer to permissive cells and the biological effect of the transferred gene. In this study, we developed a quantitative cell-based potency assay and utilized it as a screening tool to optimize various process parameters, such as plasmids ratio, transfection reagents, feeding scheme and culturing conditions, during the early stages of process setup. These different parameters are tested on a smaller scale in culture vessels or bioreactor system like the Ambr® 15 to maximize the throughput. To compare the *in vitro* potency of rAAV generated from each condition, we used green fluorescent protein (GFP) as gene of interest to have a visual readout for the quantification of transgene expression. Following a cell lysis step on the whole culture with detergent-free buffer, crude rAAV2-GFP samples generated from transfected HEK293 suspension cells were prepared in serial dilution to transduce HEK293 cells at the 96-well scale. GFP signals were visualized and captured 72 hours after transduction using Incucyte® S3 Live-Cell Analysis System. The percentage of GFP-positive cells was quantified via automated cell identification after specific analysis masks were optimized for HEK293 cells. Our results show dose-response relationship between the GFP-positive cell population and virus inoculum with intra- and inter-plate reproducibility. In addition, we demonstrate how this robust assay can be applied in the process parameter optimization.

P171

Application of CuO-CymR inducible system in gene therapy viral vector manufacturing

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In gene therapy projects, the gene of interest (GOI) has impacts on viral vector yield and quality. GOI may be toxic to vector packaging cells, disrupting viral replication, packaging, and result in low yield. We compared multiple inducible system to inhibit GOI expression during packaging and thus rescue the yield. CuO-CymR system stands out during head-to-head comparison. CuO is a short regulatory element, which is inserted upstream of the GOI coding region and binds to CymR. By exogenous expression of CymR in packaging cell, they inhibit GOI expression. The inhibition is promoter independent but affected by the location of CuO. The vector gene expression is not influenced by CuO insertion. The vector performance is comparable to the version without CuO

element for both *in vitro* and *in vivo* studies. We tested a panel of GOIs which produce extremely low yield by default packaging system, most of them have 10-50 folds increased yield by introducing CuO-CymR system. Importantly, this system could apply to not only rAAV but also lentiviral vector (LV) and other vectors for manufacturing. We scaled up LV production in the industrial manufacturing system, which solved the problem of low productivity, and the purified vector function as expected.

P172

A novel cell-based AAV RNA-sensing infectivity reporter assay

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1: Teknova

A current limitation in AAV development is the lack of standardized and universal cell-based assays that provide insight into infectivity. Utilization of GFP as a construct proxy is limited in its direct applicability towards the final drug substance and TCID-50 based approaches are laborious, costly, and exhibit extensive measurement variability. Here we demonstrate a novel cell-based infectivity assay that measures the ability of the AAV to infect HEK293 cells. The assay uses a novel method for tagging AAV transgene's RNA and results in the expression of a fluorescent protein reporter upon transgene transcription. A 125 bp RNA tag is generated by placing a new RNA sensor module (non-coding RNA) into the post-transcriptional terminator region of the transgene. We exploit the molecular mechanism RNA pol-II transcriptional elongation beyond the terminator sequence to concurrently transcribe non-coding RNA (ncRNA) with the coding sequence of the upstream gene. As a next step, the ncRNA gets processed by the genetic machinery encoded in the engineered HEK293 cell and mediates the activation of the fluorescent reporter in a manner that reflects the activity of the gene of interest (GOI). Adding an ncRNA sequence does not involve changes to the GOI coding sequence, therefore it does not alter the packaging, regulation, or stability of GOI expression. Our data demonstrate that novel infectivity reporter assay can significantly facilitate improvements in process development workflows for both upstream and downstream optimization.

P173

Optimization of AAV sample preparation towards accurate quantification of viral titer by PCR

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For AAV applications ranging from research tools to clinical-stage gene therapeutic strategies, the ability to quantify AAV vector concentration precisely and reproducibly is crucial. While several analytical methods are available, digital polymerase chain reaction (dPCR) is one of the most robust and reliable ways to determine absolute quantification of viral DNA at various stages of production. To ensure accurate titer measurements by dPCR from transgene DNA, unpurified AAV samples require DNase-I treatment to remove exogenous DNA prior to capsid disruption. Multiple

factors, such as EDTA concentration and the DNase activation and inactivation method, can significantly impair the amplification efficiency of the protected viral vector genome. This highlights the potential for large discrepancies between the results of different laboratories using analogous protocols. In this study, using a design of experiments approach, we have extrapolated an optimal DNase-I application that can be used as a standard dPCR treatment for AAVs. We determined the best concentration of EDTA to allow the inactivation of DNase-I, while not inhibiting the dPCR reaction following the treatment. Based on our findings, we have established a select protocol for processing AAV samples prior to dPCR. This protocol has shown robustness across serotypes, PCR platforms, and production workflows (from harvest to polishing steps). Additionally, a unique buffer formulation was optimized for sample dilution of an AAV sample prior to performing PCR analysis. This enhanced protocol allows faster and more robust analysis of vector genomes for AAV analytics.

P174

End-to-end solutions for AAV production, purification, and analytics

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The developed tropism of Adeno-Associated Viruses (AAV) allows them to target human-specific tissues, conferring AAV therapeutics with the ability to become primary vectors for gene delivery. However, due to the inherent nature of differences in transgene and capsid design, many considerations are required to achieve high recovery and purity at each stage of the bioproduction workflow. In particular, emphasis on optimized downstream processing (including filtration, TFF, capture, and polishing) is required across serotypes and transgenes. Here we have developed an end-to-end solution that includes AAV production, purification, and analytics and novel, proprietary products that facilitate an accelerated timeline to the therapeutic market. The list of our products include AEX Buffer Screening Kits, Affinity Buffer, PCR Sample Preparation Kit, and more. To develop such products, we have designed implemented numerous design of experiments (DOE), utilizing multiple process parameters (including pH, numerous elution salts, excipients, surfactants, stabilizers, osmolytes, enzymes, additives, extraction methods, etc). A unique feature of our products is that they provide multiple formulation compositions, allowing selection of the top candidate based on raw material needs, grade, cost, and supply access and the ability to scale to GMP grade materials.

P175

Long-term and accelerated stability of an AAV8-based gene therapy

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Gene therapy has emerged in the 21st century as the most promising technology for the treatment of severe diseases. The European Medicines Agency (EMA) defines a gene therapy product medicinal product (GTMPs) as: *"a vector or delivery formulation/system containing a genetic construct engineered to express a specific transgene ('therapeutic sequence') for the regulation, repair, replacement, addition, or deletion of a genetic sequence."* Adeno-associated viruses are widely used for gene delivery as its clinical efficacy and safety have been demonstrated. Recent advances in gene therapy have led to the development of treatments for a wide range of diseases such as rare genetic diseases, congenital disorders, cancers, degenerative diseases, and autoimmune diseases. Over the past 20 years, more than 30 therapies have received marketing authorization (Zolgensma®, Gendicine®, Abecma®, …) and currently, several dozen gene therapies are being tested in clinical trials worldwide.

Prior to human use in clinical trials, the stability profile of the drug product should be assessed in accordance with the Q5C guidelines issued by the ICH. The stability studies aim to demonstrate that the physicochemical, biological, and microbiological properties of the drug product are not altered under the influence of various environmental factors, such as temperature, throughout the shelf-life to support a stable safety product profile for administration.

The current work addresses this issue with an rAAV8 vector carrying a modified genome containing the cDNA sequence encoding for the human uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) enzyme involved in the Crigler -Najjar syndrome.

Long-term (-80°C), accelerated (+25°C) and forced-degradation (freeze/thaw) stability studies were performed. To generate supportive data on the AAV8 drug product shelf-life, the long-term stability study was conducted for 5 years. The following quality attributes were evaluated to establish the quality profile of the drug product: vector particle concentration, infectious genome titer, purity, expression assay, aggregation, and pH.

Data from stability studies showed no significant variation in the quality attributes tested for the AAV8 drug product, hence demonstrating a good product stability and safety for the patient. Moreover, based on available long-term stability data, a shelf life of 5 years was assigned to the AAV8 drug product.

P176

Advances in cell and gene therapy cell microarray screening

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Cell and gene therapies are booming, with six CAR-T cell therapies approved by the FDA and AAV vectors being used in over 200 clinical trials in 2019. However, with AAV gene therapy challenges to overcome organ specificity, innate immunity and dose-dependent toxicity remain. CAR-T cell therapy must address side effects, such as cytokine release syndrome, high relapse rate and efficacy against solid tumours. Here we describe development of screening capabilities in cell and gene therapy using the Charles River Retrogenix Cell microarray screening technology, to sustain this growing market globally.

The Retrogenix cell microarray library contains over 6,500 full length human cell surface, secreted and heterodimeric proteins that are over-expressed in Human Embryonic Kidney-293 (HEK293) cells and screened for interactions with test molecules. This technology can be used on a wide range of complex ligands to identify primary receptors and potential off-targets, which widens the scope for the development of vaccines and new therapies. We demonstrate our ability to screen cell therapeutics and identify CAR-specific interactions and non-specific T-cell interactors.

This technology incorporates the use of proteins in their natural environment, elevating high specificity with rapid turnaround, generating high data quality with low false positive and negative rates. Charles River has a lot of experience in this space with an increasing client portfolio, by their capability to offer bespoke testing packages for different project needs. This resulted in development of cell microarray screening capabilities for diverse therapeutic modalities, including cell therapeutics and AAV's.

P178

Directed Evolution of Adeno-Associated Virus Variants Enabling Potent Gene Delivery for the Treatment of Osteoarthritis

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Osteoarthritis is characterized by the loss of articular cartilage and is a representative degenerative disease with a very high incidence. Despite the great promise of cell therapy for the treatment of osteoarthritis, which involves injecting potent cells to differentiate directly into cartilage, the currently available methods pose several limitations, such as the loss of transplanted cells and low chondrogenic differentiation efficacy. Various gene therapy strategies using gene delivery vectors such as virus, nanoparticle are applied to treatment of osteoarthritis due to these limitations. Among these delivery vectors, adeno-associated virus (AAV) vector is in the spotlight in many gene therapy applications due to its advantages, such as high efficiency and safety. In this

study, we aimed to develop AAV-based gene therapy approaches that can promote osteochondral regeneration. Many AAV serotypes have been identified with different tissue tropisms, which is probably determined by cell surface receptors. Thus, we produced the AAV2, AAV5, AAV8, AAV9 viruses carrying a GFP reporter gene and then compared their infectivity in human chondrocyte cells and other cell types. It was demonstrated that the wildtype AAVs showed moderate transduction efficiency in those cells, it would not be sufficient enough to achieve selective and effective gene delivery to target tissues. Therefore, we developed a AAV8 peptide library by utilizing the directed evolution techniques for capsid engineering. We intend to prepare an AAV drug delivery tool that can maximize the transduction efficiency to cartilage cells, which are desired target organs.

P179

Verification of Full-Empty Ratio of PROGENs Gold Standards with Orthogonal Methods

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Today, AAV-based gene therapy is one of the most promising and fastest growing branches of modern medicine. Consequently, there has been a rapid increase in new methods and technologies. However, the variety of available orthogonal methods e.g. for the determination of the full vs. empty ratio of AAV preparations has raised questions regarding the comparability of obtained results using different methods, and which methods ensure a comprehensive and reliable output. To verify the different methods and to evaluate the comparability of the results, highly characterized standard materials are indispensable.

PROGEN has established a comprehensive characterization process for our internal gold standard material based on the published procedure for the international ATCC standard material. In this process, the final calculation of the total capsid titer is based on the determination of the filling grade by negative staining electron microscopy (EM) and the genome titer by qPCR. PROGEN's AAV ELISAs are calibrated using these highly characterized internal gold standards. They are a high accurate total capsid titer determination method, that can be used throughout the entire development and manufacturing process.

Over time new characterization methods have entered the AAV gene therapy field enabling growth in our portfolio of characterization methods.

Here, we present our study on current orthogonal methods for determining the full/empty ratio and their comparability using PROGEN’s established gold standard material data. The study includes the full empty analysis using the current gold standard method, analytical ultracentrifugation (AUC), as well as more recent methods such as charge detection mass spectrometry (CDMS) and mass photometry (MP) in comparison to our EM results obtained with negative staining EM.

P180

Total and Encapsidated Residual DNA in AAV production

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Regulatory agencies require the evaluation of residual DNA and recommend the evaluation of encapsidated DNA in AAV products. Those are considered as quality attributes essential to ensure the safety of a viral vector. Therefore, appropriate methods must be developed to assess them.

To date, there is no clear consensus about neither the methodology to apply nor the acceptance criteria for the residual DNA, defining that the measurement should be done by suitable methods and that the DNA levels in the final product should be as low as possible and the acceptance criteria established by the manufacturer should be justified.

In terms of the residual plasmid DNA, one of the main risks derives from the presence in the final product of the antibiotic resistance cassette. Moreover, it has to be considered that the plasmid can come from any of the helper plasmids used during the manufacturing process. Noteworthy, the presence of antibiotic resistance sequences can be as high as 6% in certain AAV manufacturing platforms.

As for the residual host cell DNA (HCD), there are several considerations: 1) the total residual DNA levels; 2) The DNA size should be shorter than 200bp: 3) the absence of viral oncogenic sequences from the host cell genome, as for example the E1A/B gene. A final consideration relies on the difference between the total residual DNA and the encapsidated DNA, as the latter shows a higher chance to reach the DNA of the patient receiving the viral vector therapy with the associated risk of producing adverse effects in the individual.

As part of AAV ExoReady® Platform, we have developed an integrated methodology to evaluate total residual DNA and encapsidated residual DNA on AAV2 and AAV8 products (expandable to the other serotypes) using ddPCR as a workhorse. A multiplex reaction is used to quantify the content of DNA from either the cell line or the plasmid in different material including the VR-1616 AAV2 reference standard, two other commercial controls (AAV2 and AAV8), or samples at different stages of the downstream purification process. A divergent pre-treatment approach allowed us to efficiently measure the presence of contaminant DNA outside and within the viral particles. The presence of host cell DNA was observed mainly as free residual DNA (non-encapsidated). E1A/B sequences were barely detected, and their assessment will require the measurement of several reactions. The main encapsidated contaminant originates from plasmid sequences although the presence of the antibiotic resistance cassette was limited.

Altogether, the residual quantification package developed within ExoReady® provides a broad vision of the presence and quantity of residual DNA in viral vector containing samples which can guide downstream process and improve safety of the product.

P181

Multiplexed comparison of rationally-designed AAV CNS capsids in vivo

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The limited ability of AAV capsids to cross the blood-brain barrier after systemic administration, together with the high sequestration in peripheral organs constitute major limiting factors for CNS-directed AAV-based gene therapies. Therefore, engineering novel AAV capsids that can overcome this barrier and efficiently and specifically target the CNS cells constitute a very active field of research.

Here we have produced 26 rationally-designed capsids by mutating and/or inserting short peptides in either the variable region (VR) IV and/or VIII of the AAV5 or AAV9 capsid gene. The capsid pool was systemically delivered (intravenously) to 7-week-old mice to identify variants efficiently and specifically transducing brain cells. Thanks to the barcoded reporter transgene, the biodistribution and transduction of each capsid variant could be traced at both DNA and RNA levels using next-generation sequencing and single nuclei RNA sequencing (snRNAseq) to determine cell tropism.

Overall, six variants exhibited significantly enhanced transgene expression in the brain compared to AAV9, and snRNAseq results confirmed that transgene expression was primarily neuron-specific. Notably, CapX-38 showed up to 219-fold increase in brain transduction compared to AAV9, while CapX-25, which also harbors a modification in VR-IV, additionally showed 37-fold decreased liver transduction. These results were further validated in single-plex manner using both qPCR and histology readouts.

Overall, this method enables the rapid variant stratification and identification of the most promising candidates for CNS applications and emphasizes the critical role of both VR IV and VIII for brain targeting and liver detargeting capsid engineering.

P182

Establishment and optimization of suspension adapted HEK293 cell line for high yield production of novel blood brain penetrant capsids

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There is a need for improved viral vector production technology to meet the demand of best-in-class gene therapies in early-stage development. Novel AAV capsids generated from Voyager's platform hold promise to target desired cells and are shown to penetrate the blood-brain barrier in non-human primates and achieve widespread transgene expression that is

superior to conventional AAV serotypes. In this poster, we discuss the establishment of our own cell line (VYGR-293) by adapting an adherent serum-fed culture to a suspension-based serum free culture. This cell line was used to evaluate the productivity and product quality of several novel AAV capsids in a transient triple transfection process. The transfection process was further developed to identify the optimal transfection reagent and plasmid ratio (Helper: Rep-Cap: Transgene) per cell using Design of Experiment (DOE) studies. Further optimization such as evaluating multiple plasmid ratios, varying cell densities at transfection were performed to improve AAV productivity. In conclusion, we demonstrate the capability of the newly developed cell line to produce high quality AAV to improve the delivery of innovative gene therapies.

P183

Enhancement of AAV-mediated gene transfer for bone regeneration in vitro using cationic poloxamers

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AAV is a promising candidate for gene therapy since it provides prolonged transgene expression without the risk of insertional mutagenesis. This can be used in the context of bone regeneration by packing genes into the AAV vector that promote the formation of new bone. In order to improve gene expression, enhancers which are compatible with in vivo and potential clinical use were developed and tested in vitro.

Different newly developed proprietary cationic poloxamers were combined with AAV vectors and were tested for reporter gene expression of cell lines and primary cells upon AAV-transduction in vitro. The mixes included thermoresponsive hydrogels. The polymers and serotype with the best results were selected for subsequent experiments using bone marrow-derived ovine mesenchymal stem cells. The viral vector was either loaded with the reporter gene eGFP or with the growth factor genes BMP-2 or VEGF. Transduction efficiency was evaluated via fluorescence measurements (eGFP) or ELISA (growth factors). Cell viability was determined by XTT assay.

The experiments revealed a cationic poloxamer, which not only increased transduction efficiency in a dose dependent manner, but also promoted cell survival. Enhancement was seen with AAV2, AAV6 and AAV8 capsids, albeit with different rates. Cationic poloxamer additionally protected AAV from drying, potentially allowing for lyophylization. Thus, a cationic poloxamer with potential for in vivo application and clinical use was identified, which enhanced AAV-mediated gene transfer in general and in cells relevant to bone regeneration.

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P184

Screening out the noise: removing contamination tridents from capsid screens

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In the search for next generation Adeno-associated virus (AAV) capsids for gene therapies, large-scale directed evolution experiments are common. In the pursuit of finding rare capsids with enhanced properties, high library complexity is often desirable: Buying more tickets for the tropism lottery increases the probability of winning. However, elevated complexity results in less replicability across samples, and increases the relative impact of index hopping for multiplexed Illumina samples.

Index hopping effectively causes contamination at the sequencing stage with reads misassigned to incorrect samples. With combinatorial indexing, up to 1% of reads from sample A can be read as being from sample B. When the complexity of the library is orders of magnitude greater than 100 capsids, this 1% rate can easily cause spurious trends in the data, and lead to sub-optimal capsid selection.

With very high complexity, where you expect poor capsid overlap between samples, index hopping can lead to top performing capsids in a given sample leaking into multiple samples from other organs, where the capsid is not actually present. This pattern can spuriously imply consistent performance in these other organs of a likely useless capsid.

We have found the characteristic pattern of this kind of contamination is an unusual “trident” pattern correlation, which forewarns of these issues. We show unique dual indexing can massively reduce the rate of index hopping, and characterisation of these rates may provide essential context to capsid library screening data.

P185

Advancements in Adeno-Associated Virus Bioprocess Development: Enhancing Chromatographic Strategies for Efficient Gene Therapy Production

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Gene therapy utilizing adeno-associated virus (AAV) vectors has emerged as a promising therapeutic modality for the treatment of genetic and acquired diseases. To meet the demands of scalable and efficient AAV production, this study focused on the development of chromatographic steps in the bioprocess. Specifically, the activities encompassed performing a Design of

Experiments (DOE) to optimize the capture chromatography step, evaluating different chemistries for the Polishing chromatography step, conducting a 1L confirmation study, benchmarking against existing methodologies, and incorporating analytical techniques for process monitoring.

In the initial phase of the study, a DOE was performed to develop an optimized capture chromatography step for AAV purification. Key parameters such as pH were systematically varied to identify the optimal conditions that resulted in maximum AAV recovery and purity. The DOE approach facilitated efficient process development and enhanced the understanding of critical process parameters.

Furthermore, three different chromatographic chemistries were evaluated for the polishing step, aiming to establish a robust and selective purification strategy. Comparative data on the performance of these chemistries is presented, including overall AAV yield and purity (ratio of full particles). The findings provided insights into the most suitable chromatographic medium for AAV polishing, ensuring the removal of product impurities and the attainment of high product quality.

To validate the scalability and reproducibility of the optimized process, a confirmation study was conducted at 1L scale. This study provided important insights into the performance of the developed chromatographic steps at larger scales, paving the way for future process scale-up.

Benchmarking against existing methodologies was performed to assess the efficiency and productivity of the developed AAV bioprocess. Comparative data is presented, highlighting the advantages and improvements achieved through the chromatographic strategies employed in this study.

Additionally, advanced analytical techniques were incorporated in process development and monitoring. This included the use of high-resolution liquid chromatography, quantitative PCR, ELISA and other relevant analytical tools to ensure accurate characterization of AAV vectors throughout the bioprocess.

In summary, this research emphasizes the crucial role of chromatographic strategies in the development of efficient AAV bioprocesses for gene therapy production. The outcomes of this study contribute to the advancement of AAV production methodologies, facilitating the translation of gene therapy into effective clinical treatments.

P186

Characterisation of the human humoral immune response against adeno-associated virus 2 (AAV2)

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Adeno-associated virus (AAV) vector-based gene therapies have many advantages such as broad tissue tropism, non-pathogenicity, and long-term expression. However, there are therapy-limiting factors which prevent several patients from receiving AAV vector-based gene therapy. One of these limiting factors is the pre-exposure to AAV leading to seroconversion. Particular frequent in the human population are neutralising antibodies (nAb) against AAV serotype 2 (AAV2). As of today, the humoral immune response against AAV2 as well as respective monoclonal nAbs have not been well characterised. Moreover, structural characterisation of AAV2 epitopes targeted by

nAbs is a prerequisite for efforts to engineer capsids which escape antibody-mediated neutralisation.

Here, we characterised the human humoral immune response against AAV2 in human sera of healthy donors using enzyme-linked immunosorbent assay (ELISA), followed by AAV2-specific memory B cell sorting using fluorescence-activated cell sorting (FACS). Interestingly, we detected in only 22.7 % of the 22 samples from random blood donors anti-AAV2 specific IgG titers. From these, we established a sorting scheme for AAV2-specific memory B cells. 0.01 % of the memory B cells from donors with a positive anti-AAV2 titer were found to be AAV2-specific in FACS, detected by an optimised anti-AAV2 antibody A20. A20 was improved regarding its affinity and stability using computational antibody design. The now isolated memory B cells will be used for paired single cell sequencing to obtain heavy and light chain sequence information with 10X genomics. In conclusion, our study will reveal and characterise the humoral immune response against AAV2 and further identify specific epitopes in the capsid that might be targeted by nAbs. This will inform AAV capsid engineering and design in the future.

P187

Highly specific AAV subtypes infecting hair cells and supporting cells in the mammalian cochlea

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Hereditary hearing loss impacts about 1 in 500 newborns worldwide and accounts for 50–60% of all deafness. With over 200 deafness-related genes being identified, most of these genes are expressed in the hair cells (HCs) and supporting cells (SCs) of the cochlea. The first cochlear gene therapy studies involved a limited number of capsids, most notably adeno-associated virus (AAV). AAV-mediated gene therapy has emerged as a promising therapeutic strategy that has been successfully implemented in numerous mouse models of hearing loss with remarkable efficacy to treat hereditary deafnesses. However, majority of the AAV capsids being evaluated in the cochlea have shown low transduction efficiency to the HCs and SCs of the cochlea, the cells that express most deafness genes. Here, we rationally engineered the capsid protein of the AAV9 subtype capable of effectively infecting the hair cells of the cochlea. Fluorescent viruses packaged with different AAV mutants were also injected into the cochlea respectively. One of the variants (termed HGHC-1) showed nearly 100% transduction efficiency of the HCs in neonatal (P0-3 and P5-7) and adult (P30) FVB mice 3-4 weeks after the injection. In order to obtain AAV serotypes highly targeting SCs of the cochlea, we also rationally designed variants based on the AAV-DJ capsid, which outperformed other AAV variants in transducing the SCs of the cochlea. Among them, HGSC-6 exhibited the highest infectivity (up to 90%) against SCs 3-4 weeks after the injection into P0-3 and P5-7 neonatal FVB mice. Taken together, we have identified two vectors (HGSC-6 for SCs and HGHC-1 for HCs) with promise for clinical treatment of hereditary hearing loss, and we demonstrate, for the first time, nearly 100% transduction efficacy of the HCs in mice. Overall, our findings provide a superior delivery tool for the treatment of HCs- and SCs-associated hearing loss, and our data warrant further investigations of these vectors as the potential candidates for cochlear gene therapies.

P188

Validation of a ddPCR protocol for the quantification of AAV genomes in purified vector samples

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Adeno-associated virus (AAV) is a non-pathogenic virus that is used as a delivery vehicle to transfer therapeutic genes into a patient's cells. Accurate quantification of AAV genome copies is a key challenge. In this study, we validate a ddPCR protocol for the quantification of AAV viral genomes in purified samples according to ICH Q2(R1) guidelines. To eliminate unpackaged contaminant DNA, samples were DNAseI-treated prior to droplet formation. A total of 4 sets of quality control (QC) samples were prepared and ran 5 times to assess precision, linearity, limit of detection (LOD), limit of quantification (LOQ), range and specificity. Each QC set contained an ultra-low (5 copies/µL), low (10 copies/µL), middle (100 copies/µL), and high (5000 copies/µL) AAV2 concentration. AAV2 genome copies were quantified through ddPCR (QX200 droplet digital PCR system, Bio-Rad). For all 5 runs, the %CV of all QCs at each concentration was ≤34% (= intra-assay precision). The %CV of each QC sample between the 5 runs was ≤27% (=inter-assay precision). Excluding one outlier, the intra-assay and inter-assay precision was ≤13% and ≤14%, respectively. Linearity was shown in the range of 5 to 5000 copies/µL. The LOD and LOQ was 1.36 copies/µL and 5 copies/µL, respectively. The reportable range was determined to be 5-10 000 copies/µL. Specificity was shown by the lack of signal in no template controls (NTC) i.e. samples not containing DNA. Our ddPCR protocol allows precise, specific and reproducible quantification of AAV2 genomes in purified samples. Furthermore, this protocol will be validated for additional serotypes.

P189

Detection and quantification of distinct populations of full AAV capsids.

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Adeno-associated viruses (AAVs) are commonly used as vehicles for therapeutic gene delivery to treat various genetic disorders. High-quality AAV preparations are enriched in full or heavy capsids encompassing a full-length transgene and are largely free of process impurities, such as empty and partially filled capsids containing truncated DNA. Here, we demonstrate that the affinity-purified AAVs contain distinct populations of heavy capsids that can be separated by gradient density ultracentrifugation and quantified using a fast protein liquid chromatography system with inline UV and fluorescence detection. We further show that the abundance and properties of these heavy capsid variants—such as aggregation or viral protein composition—vary with the cell production system, purification process, capsid serotype, and transgene length, which may have implications for the potency and safety of these vectors. Our results highlight heterogeneity in AAV preparations and illustrate the need for continued development of high-resolution purification and characterization methods for generating ultra-pure AAV material.

P191

Accelerating process development by establishing drug substance development platform

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uniQure delivers gene therapy through an adeno-associated virus vector-based technology and has established a platform approach to accelerate the process development. Our recent achievement is the approval of hemophilia B gene therapy. The established platform approach is implemented in multiple programs, including Huntington's disease and Fabry disease.

uniQure has a manufacturing platform with modules for starting materials, USP, DSP, drug product and analytics. This abstract covers both the USP and DSP module, which is composed of several operational units covering a range from high-throughput miniaturized scale to bench and pilot scale and to commercial production scale. Furthermore, the usage of the Design of Experiment (DoE) methodology, together with the above-mentioned operational units allows uniQure to establish a flexible, and fast-paced scientific-driven approach for drug substance process development. The established approach has been implemented in several research programs at both early and late stage and is robust in (1) delivering products with a doubled genome copy concentration in the filtered crude lysed bulk as compared to the non-optimized process, (2) maintaining a drug substance recovery yield at around 70% in the purification process for various types of gene therapies, and (3) shortening the drug substance process development timeline to 3 months for Toxicity study. In summary, a robust, efficient and more cost-effective process development can be achieved with drug substance platform available at uniQure.

P193

Platform development for AAV manufacturing with the Baculovirus Expression Vector System (BEVS)

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uniQure focuses on adeno-associated virus (AAV)-based gene therapy as a basis for one-time treatment of diseases with high unmet medical need, through a multi-module AAV manufacturing platform supported by in-house commercial manufacturing. The biological starting materials module within uniQure's AAV manufacturing platform, is based on the Baculovirus Expression Vector System (BEVS) and has been validated for commercial manufacturing. The first generation BEVS utilizes infection of insect cells with three baculoviruses, that must all infect a single cell to obtain functional AAVs. This technology is proven and effective. A second-generation BEVS has been developed and is ready for large-scale GMP manufacturing. This second-generation BEVS is based on dual infection with baculoviruses and offers improved process performance. A third-generation BEVS is currently in early development. It utilizes single baculovirus infection of a genetically modified insect cell line. This single infection module has tremendous potential for a simplified and more robust AAV manufacturing process. With this and other manufacturing platform improvements, uniQure aims to achieve faster time to clinic for higher quality

therapeutic AAVs, at 100-fold lower costs. Design and configuration of the biological starting materials is a major contributor to this aim.

P194

Safer AAV through reduced host-cell DNA encapsidation: the next generation of ELEVECTA's fully stable AAV producer cell lines

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The ELEVECTA platform yields highly productive, fully scalable, and inducible stable rAAV producer cell lines, which support robust and high-yield rAAV manufacturing. With ELEVECTA, there is no more need for transient transfection or helper virus infection. Most recently, we have upgraded our platform to further enhance rAAV safety by dramatically reducing host-cell DNA encapsidation. While most hcDNA molecules outside AAV particles can be purified away during DSP, any encapsidated hcDNA molecule is substantially invisible to the downstream process — the desired rAAV product is simply too indistinguishable from its undesired molecular variant that carries hcDNA alongside the vector genome. Also, the unavoidable "empty" or "partial" capsids in the final product may well contain hcDNA too. Any impurity is cause for concern, but encapsidated impurities are especially likely to being delivered directly into the patients' cells and their nuclei. The nuclear delivery of DNA impurities is of great concern because only in the cell nucleus can DNA impurities act both immunogenic and oncogenic. We have tackled this important concern by drastically reducing hcDNA co-packaging below quantification limits. Intriguingly, this reduction works equally well under both stable and transient AAV production settings.

P195

Assessment of synthetic double stranded DNA as alternative to plasmid DNA in AAV2 production process

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1: Exothera SA 2: TAAV

neDNA™[1] is synthetic DNA with a structure of linear double stranded and covalently closed. The enzymatic production process presents several advantages over plasmids: high and reproducible yields, industrial scalability, and batch homogeneity. Moreover, neDNA are associated with increased safety compared to traditional plasmid DNA, as no antibiotic resistance genes are needed, and no bacteria are involved in the production process, presenting regulatory advantages. neDNA material was provided by TAAV, a CDMO specialized in the critical starting material for rAAV-based gene therapies.

In this study, neDNA was evaluated as critical starting materials in manufacturing for recombinant adeno-associated virus (rAAV) production. The efficiency of neDNA was assessed in a triple transfection process to produce AAV2 in HEK293 cells. The performance was compared to the standard plasmid using the AAV manufacturing process from the exoREADY™ platform. A Design of experiment (DoE) approach was used at shake-flask scale to determine optimal transfection parameters: transfection reagent, DNA concentration per million of cells and ratio between neDNA coding for gene of interest, gene of AAV2 REP/CAP and gene for helper. An optimum condition was identified, associated with an estimated genomic titer and full/empty ratio similar to the one obtained with reference plasmids. The optimal process was scale-up in 1 L bioreactor and titer and full/empty ratio were slightly increased compared to flask-scale. In addition, material has been processed in downstream to evaluate impact of neDNA on purification yield and residual impurities. Results highlighted expected recoveries and impurity clearance along purification stream.

The study demonstrated that neDNA can be an efficient alternative to plasmid DNA with improved safety without impacting production yield. In conclusion, our findings suggest that neDNA plasmids are a viable option for AAV manufacturing. Their implementation can potentially streamline the safer production process, leading to high yields and enhanced therapeutic efficacy of AAV-based gene therapies.

[1] Technology for making neDNA™ is licensed from Touchlight IP Ltd.

P196

Optimizing AAV Production in Insect Cells in a Chemically Defined Media Platform

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Clinical gene therapy has a crucial need for efficient, scalable and cost-effective viral vector manufacturing that will enable developers to bring therapies forward. Adeno-associated virus (AAV) is a non-enveloped virus which can be engineered to deliver DNA to target cells for gene therapies. Due to its wide host-cell range, transduction efficiency and longer lasting transgene expression, it is quickly becoming a vector of choice. Additionally, the Sf9 (*Spodoptera frugiperda*) insect cell system along with baculovirus is a popular platform for large-scale production of recombinant AAV (rAAV). As a result of the lack of chemically defined medium formulations and presence of poorly-defined hydrolysates, developers often experience lot-to-lot inconsistency and variable doubling times with each passage. In this study, we evaluated the use of a chemically defined, animal component-free cell culture medium, that supports production of a standard AAV (AAV2) and an engineered AAV (Anc80), while allowing for rapid adaptation from a variety of starting media. Cultures rapidly achieve a stable doubling time of around 24 hours that maintains for more than 45 passages. Cell densities of over $3x10^6$ cells/mL were achieved by three days after seeding and yielded titers as high as $2.5x10^6$ vg/mL, providing faster cell growth rates and higher AAV titers than other media evaluated. Additionally, most of the AAV particles are secreted, allowing for simplified purification. Metabolite analysis of spent media showed low lactate and ammonia production indicating better overall cell and virus health. This chemically defined, hydrolysate-free, medium has significant benefits for gene therapy applications producing AAV using insect cells.

P197

Enhancement of rAAV-2 viral vector yield recovery in upstream lysate material

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1: Pharmaron

Production of certain AAV serotypes for use as gene therapy vectors requires a cell lysis step to release capsids that are not secreted from the producer cell line. Cell lysis also releases host cell impurities that may interact with the capsid itself or other production vessel components to form large aggregates that sequester the vector, negatively impacting yield following lysate clarification. At Pharmaron, driving innovation via our state of the art high-throughput upstream capabilities and advanced analytical tools, various pre-clarification treatments were tested to help overcome this recombinant AAV production process challenge. The high-throughput Ambr250 bioreactor system was used to simultaneously trial five treatments in various combinations. A specific combination of two of these treatments increased capsid titres by more than two-fold compared to untreated lysate and resulted in a several-fold increase in post-clarification yield. Nanoparticle Tracking Analysis (NTA) demonstrated an overall reduction in the prevalence of large aggregates following post-lysis treatment, consistent with the increased yields observed. This study demonstrates the effective application of Pharmaron's high-throughput process development platforms, Design of Experiments expertise and state of the art analytical technologies to solve critical process challenges.

P198

Scalable Production of AAV from shake flasks to 1000L single use bioreactors using the Gibco™ CTS™ AAV-MAX Production System

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1: Thermo Fisher Scientific

Production of high-titer AAV at clinical scale remains a significant challenge for the gene therapy industry. Developers need to understand how to optimize, scale, and qualify their manufacturing process for AAV production under the tightest of timelines. Due to high demand in the gene therapy space, effective solutions to address these challenges must be developed and implemented. Until recently, AAV production took place primarily in adherent cultures using 293T cells in the presence of fetal bovine serum; however, such adherent systems suffer from significant drawbacks including difficulty in scaling up, the presence of the SV40 large T antigen in the producer cell line as well as cost, consistency and regulatory considerations stemming from the use of animal sera. To address these shortcomings, we present data on the Gibco™ CTS™ AAV-MAX Helper-Free AAV Production System, a chemically defined, suspension based AAV production system that allows for scalable, high titer production of AAV viral vectors in a non-293T cell lineage. The AAV-MAX system comprises all of the components required for scalable AAV production in mammalian cells including: (1) a clonally-documented, high-titer 293F-derived producer cell line, (2) a chemically defined growth and expression medium, (3) a production enhancer, (4) a cationic lipid-based transfection reagent and booster, (5) a protein-free complexation buffer, and (6) a Polysorbate 20-based lysis buffer. Through a series of case studies,

we demonstrate upstream and downstream approaches for consistent and scalable production, purification, and characterization of AAV at scales ranging from shake flasks to 50L and 1000L single use bioreactors.

P199

Accelerate AAV characterization and process development with the mass photometry technology

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While adeno-associated virus (AAV) has emerged as a leading vector for gene therapies, there is still an urgent need to improve AAV associated analytics. Indeed, AAV manufacturing processes generate empty capsids, which greatly complicate vector purification (downstream process) and affect product safety and quality. Several analytical techniques have been established for the AAV empty/full ratio, however they still face great limitations such as cost, accuracy, sensitivity, lead time and sample consumption. Mass photometry is an ideal technology applied for AAV analytics, as it enables users to precisely measure the empty/full capsid ratio for AAVs of any serotype using very low sample volumes and concentrations. Here we show how mass photometry can be used to characterize AAV capsids during AAV purification and how this technology can provide capital advantages for AAV process development.

P200

Improving AAV vectors via chemical engineering using the AAV-Ligand conjugation platform (ALIGATER™)

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Recombinant Adeno-Associated Viruses (rAAV) are the most promising vectors to treat monogenic diseases or some idiopathic disorders. Lately, genetic variants of AAV vectors have been generated, either by library screening or by rational design, to improve tissue specificity and limit off-target transduction. However, depending on the route of administration, their effectiveness was often counterbalanced by their limited spreading, off-target accumulation, or immunogenicity. To address these different issues, we used an innovative engineering approach to chemically modify and improve existing AAV capsids properties.

Coave Therapeutics' proprietary AAV engineering platform, called ALIGATER™, uses a rational design approach to conjugate selected functionalizing moieties to specific amino acids at the surface of the capsids. The ligands include small molecules, peptides or aptamers, which are covalently linked to AAVs to improve tissue spreading, and cell transduction. We previously showed that the first-generation conjugated AAVs (coAAVs), modified with sugar-based ligands, outperformed traditional AAVs in the brain of mice, rats and NHPs after a local injection.

The conjugation step does not require additional process development. It can be performed on existing AAV products or it can be easily implemented within an existing downstream process (DSP). It does not cause any loss of AAV material, and the unbound ligands can be completely removed to match manufacturing requirements. It was successfully performed on multiple serotypes (natural or engineered) produced through different processes (transient transfection, baculovirus/Sf9 system).

An analytical toolbox with orthogonal methods is being applied to support our technology and the batch-to-batch reproducible signatures of AAVs. The present work focus on orthogonal methods development to further characterised our conjugated AAV (coAAV) as well as ligand related impurities.

Conjugated AAV thus represent a credible alternative to overcome several AAV related bottlenecks, with an easy manufacturability that may be implemented within any process development and CMC groups.

P201

Adeno-associated virus (AAV) delivery system using cat echol for immune evasion

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1: Yousei University

The immune response poses challenges to the efficiency and safety of adeno associated virus (AAV) research in gene therapy. This research investigates the significance of immune evasion strategies in AAV research, drawing insights from FDA approved therapies. When viruses are injected, the antibody response can hinder treatment efficiency and trigger excessive immune inflammatory reactions in patients. In cases where the therapeutic effect is diminished, repeated injections become necessary, necessitating the development of immune response control technologies. AAV and compounds containing catechol groups can form complexes due to the strong binding affinity between the catechol group and the AAV capsid proteins. The catechol groups act as bridging molecules, facilitating the attachment of tannic acid to the AAV particles. The formation of complexes between AAV and compounds with catechol groups offers a range of advantages, including immune evasion, enhanced stability and improved transduction efficiency. Firstly, compounds with catechol groups provides a protective coating around the AAV vectors, shielding them from neutralizing antibodies present in the immune system. Furthermore, AAV/catechol complexes have been shown to evade human immunoglobulin G (h IgG) and anti AAV2 antibody (A20), preventing the recognition of viral particles by neutralizing antibodies. This immune evasion property helps to prolong the circulation time of AAV vectors, increasing their chances of reaching target cells and exerting their therapeutic effects. These advantages contribute to the development of more effective and efficient AAV based gene therapies, with the potential to improve treatment outcomes in various diseases and conditions. Ongoing research continues to explore the optimization of AAV/catechol complexes to improve their efficacy, safety, and delivery efficiency in various gene therapy applications.

P202

Novel engineered plasmids and optimized HEK293 cell line improves AAV productivity

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The need for AAV-based therapies necessitates the development of a robust GMP manufacturing platform that can be scaled and support high titres production during the manufacturing process. Traditionally AAV manufacturing for gene therapy entailed the use of labour-intensive adherent-based HEK293 processes not amenable to scale-up. Suspension adaptation of HEK293 cells has enabled more scalable processes but remains time-consuming. Low AAV productivity and lack of reproducible, commercially viable, platform processes continue to be key challenges facing AAV gene therapy developers. Lonza has responded to these challenges by establishing a robust and scalable suspension manufacturing platform for AAV production.

P203

An experimental and computational approach to investigate risks associated with viral/vector integrations into the human genome

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Viral-derived vectors and wild-type viruses can have severe consequences when integrated into undesired genomic regions, leading to tumorigenesis, insertional mutagenesis and viral evolution and persistence in the host genome. Despite the increased concern associated with these off-target and adverse events, currently, there are limited approaches to detect viral integrations, especially at low-frequency. Recent experimental advancements, such as target enrichment sequencing, together with accurate computational detection tools, open a new avenue for biosafety assessment in cell and gene therapy. In my research, we are implementing an experimental and computational pipeline to accurately detect integrations. We have evaluated the performance of our computational approach, which relies on the VSeq-toolkit viral integration tool, on simulations and target enrichment experiments. Based on 18 simulated datasets, with different, number of integrations and integration patterns, viral/vector types, viral/vector lengths, and other sequencing and molecular challenges, we established the sensitivity and specificity of our pipeline. We find our pipeline to work well in most scenarios, with sequence coverage of integrated sites being the limiting factor. Nevertheless, we were able to investigate therapeutic vector integrations such as OTC/AAV and natural integrations of HIV-1. Also, we have investigated natural integrations of AAV2 and HHV6 in children with acute hepatitis of unknown origin. Finally, to aid scientists and clinicians in understanding adverse events following administration, integration patterns and behaviours, we report our integration analysis in a simple format, including the frequency and distribution of viral/vector integration sites in different

genomic regions, genic and intergenic. This will be part of a future service for the assessment of genomic risks in therapy.

P204

Development of full capsid enrichment step in AAV9 purification process with CIMmutus PrimaT monolithic column

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1: Genethon

In the context of process purification improvement of a gene therapy based on AAV9 product, CIMmultus PrimaT monolithic column developped by Sartorius was evaluated for the full capsid enrichment of the product. Different parameters were tested like viral loading, residence time and conductivity. Based on the generated data like the full capsids enrichment rate and the VG recovery, Prima T monolith may be used in a purification process to enrich the product in full capsid.

P205

Innovative separation sciences for accurate plasmid analysis

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1: Pharmaron

Production of high-quality plasmid DNA requires the establishment of a variety of analytical assays to monitor critical quality attributes (CQAs). Here Pharmaron provide three case studies for critical analytical assays required for critical starting material testing. Using both Ultra Performance Liquid Chromatography (UPLC) and Capillary Electrophoresis (CE) Pharmaron demonstrate how they can assess the plasmid CQAs with superior methods, offering a higher level of accuracy over traditional analytical techniques.

P206

Media development strategies to increase AAV titre at large scale

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1: Thermo Fisher Scientific 2: iBET

Adeno-associated virus (AAV) has become an attractive vector for gene therapy; however, generally low titer yield limits its viability as a therapeutic. Various methods have been employed to achieve increased titer, including cell engineering, expression platform optimization, transfection optimization, and media development.

One of the preferred methods to produce recombinant AAV (rAAV) is helper-free triple transfection using HEK293 cells. We aimed to develop media that addresses the lower titer of the HEK293 system, but the high diversity of HEK293 cell derivatives and processes used for gene therapy would make it difficult to develop one medium that suites all uses. Therefore, we developed a panel of media to support AAV production by helper-free triple transfection using HEK293 cells with increased viral titers agnostic of manufacturing processes or cell lineage.

P207

Improving Adeno-Associated Virus (AAV) vector manufacturing through process development in GMP - ready cell lines

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1: Cell Therapy Catapult

Recombinant adeno-associated virus (AAV) vectors have gained significant importance as a gene delivery method for innovative gene therapy products. However, the existing batch production process using human embryonic kidney (HEK) 293 cells suffers from inefficiency and high costs. The lack of a comprehensive understanding of the biology of host cell pathways involved in AAV production limits the optimisation potential of the production process. In the quest for an improved AAV process, we have generated GMP-ready monoclonal HEK293 cell lines from a suspension adapted HEK cell line and sought to employ them in omics studies to unravel the underlying biological processes. However, high quality data generation requires consistency across cell lines with regards to culture performance and characteristics, including growth rates, aggregation, transfection capacity. Hence, establishing comparability between cell lines is essential before performing further studies.
In this study, our focus was primarily on process development. We successfully developed a scalable, batch transient transfection process for AAV production in HEK293 cells. The operational parameters were optimized using a design of experiment (DoE) approach on the Sartorius Ambr15 system, leading to an optimised design space. The DoE model prediction was confirmed during process scale-up to the Sartorius Ambr250 system, providing comparable titres to the small-scale screening study and thus validating the optimsed process parameters. Notably, the optimised process was verified across three distinct HEK cell lines allowing productivity-based

ranking. Finally, we further adjusted cell growth rates by investigating the growth rates of the different HEK lines and subsequently modelling appropriate seeding densities to match process comparability.

In conclusion, we were able to establish a scalable batch transient transfection process for AAV production and optimise it to improve process comparability across several HEK293 suspension cell lines. The successful development of a scalable process suitable for multiple HEK293 lines has laid the foundation for future multi-omics studies aimed at uncovering host cell pathways involved in AAV production and further improving HEK bioprocessing.

P208

A novel technology for accurate quantification of viral genome and determination of lipid nanoparticle encapsulation efficiency

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1: Promega Corporation

Adeno-associated viral (AAV) vectors show great promise as a delivery system for gene therapy. However, development of robust methods for quantitation, infectivity and dose-determination has remained a challenge. Lipid nanoparticles (LNP) have also emerged as a successful delivery system as evident from the success of the COVID-19 vaccines. For LNP characterization methods for determining encapsulation efficiency remains a technical gap.

The TruTiterTM Reagent is a small molecule that is excluded from encapsulated or compartmentalized macromolecular structures like viruses with an intact protein capsid, living cells, or LNPs with intact membranes. However, the molecule covalently conjugates exposed nucleic acid and prevents subsequent amplification by PCR. This property enables selective amplification of fully encapsulated nucleic acids for accurately measuring intact AAV and LNP genome titers.

•TruTiterTM reagent is a reliable alternative compared to Dnase for consistent determination of AAV genome titers.

•TruTiterTM reagent can be used to determine LNP Encapsulation Efficiency by measuring the protected genetic payload. This technique may be especially useful in characterizing LNP with more than one gene targets.

P209

Unravelling AAV production biology by innovative proteomic approaches

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1: Cell and Gene Therapy Catapult

Currently, there is limited knowledge on the key biological pathways underpinning adeno-associated viral vector (AAV) production and how transcriptional, translational, and metabolic responses are orchestrated in human host cells. To generate cost-effective and commercially viable gene therapy products, higher AAV titres are needed (from 100- to 1000-fold yield improvement estimated to address large patients' population with high prevalence diseases) which poses a challenge to the current manufacturing capacity. Understanding the molecular events underpinning AAV production can help improving the yield and quality of the AAV products, and ultimately address the current manufacturing bottleneck, which is necessary to meet the clinical demand.

To this end, the objective of Cell and Gene Therapy Catapult is to unbiasedly identify candidate genes and cell pathways involved in the cellular response during AAV production, using a multi-omics and bioinformatics-driven approach. As part of our strategy, we aimed at applying proteomic approaches to delineate protein expression profiles of different host HEK293 producer lines during an AAV production time-course. In this study, we evaluated the use of the Olink Explore 3072 platform as high-throughput method to identify and measure the expression of up to 3000 cellular proteins.

First, we assessed the relevance of the Olink Explore 3072 platform proteomic panel for AAV production. In our study, we were able to identify 2881 proteins from the Olink panel, which allowed to cluster the samples depending on the experimental conditions and different time points of the AAV production. Further differential data analysis led to the identification of 1638 proteins which were differentially enriched across different samples and time points during AAV production. These were used for gene ontology analysis to identify key biological pathways. Finally, we confirmed the data obtained by using the Olink platform, with transcriptomic data.

Altogether, our results showed that the Olink Explore 3072 platform can be used to identify differentially expressed proteins during AAV manufacturing and can add a useful tool for identifying new molecular cues, thus contributing to enhance our biological knowledge of producer cells.

P210

Development of a novel helper plasmid: one step closer to the next generation rAAV vectors

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1: Polyplus Transfection

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 features of rAAV vectors that act on the overall size of packaged material and specificity of delivery. Here we present a novel helper plasmid pPLUS® AAV-Helper, the outcome of an extensive R&D program based on our proprietary DNA assembly e-Zyvec® technology to improve infectivity (TU/mL) and quality (full/empty ratio) of viral particles obtained through triple transfection of HEK293 cells in suspension with FectoVIR®-AAV.

P211

Next-generation transfection reagent for large scale therapeutic lentivirus production

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Lentiviral vectors are the carrier of choice for allogenic or autologous cell therapies (such as CAR-T) because of its capacity to permanently integrate viral genome into host cell DNA. To produce those vectors, cell therapy producers generally use a transient transfection system that is scaled-up during process development phases. FectoVIR®-LV is the next generation of transfection reagent, free of animal component, designed to improve LV productivity in HEK-293 cell systems. FectoVIR-LV is made for large scale manufacturing with reduced complexation volume and increased complex stability. Here we also show kindly provided preliminary data from beta-testers who evaluated FectoVIR®-LV in their cell culture systems. Miltenyi Biotec could increase by at least 3-fold functional titers compared to their standard condition, and Repligen could show that FectoVIR®-LV combined with their TFDF-intensified perfusion system led to a substantial 30-fold functional titers compared to standard batch method.

P212

Multiplex targeting of immune checkpoints with compact zinc finger-repressors to improve anti-tumor activity of T cells

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The success of engineered cell therapies, such as Chimeric Antigen Receptor (CAR)-T cells, and the continued development of genomic engineering tools point to a future of highly customized cell therapies. Despite this remarkable success, many barriers still limit broader therapeutic efficacy in solid tumors and other hematological malignancies. T cell exhaustion and an immunosuppressive tumor microenvironment represent critical constraints to maintain effector functions and achieve durable clinical efficacy. To overcome these challenges, new approaches are being developed to target negative regulators of the anti-tumor activity like immune checkpoints (ICs). Here, we describe the generation of highly specific Zinc Finger Repressors (ZF-Rs) capable of downregulating target mRNA synthesis, resulting in efficient target protein repression. This gene silencing remained stable during the entire study period (Three weeks in vitro and more than two months in vivo). Furthermore, the compact size of ZF-Rs allowed their multiplexing in a single lentiviral vector (LV) to achieve simultaneous multi-gene silencing upon a single transduction event. We also demonstrated that engineered tumor-infiltrating lymphocytes (TILs), isolated from colorectal liver metastasis (CRLM) patients, expressing PD1, PD1/LAG3, PD1/TIGIT and PD1/TIM3 ZF-Rs were able to efficiently downregulate cell surface expression of ICs and promote TILs anti-tumor activity in an in vitro killing assay. Importantly, ZF-R protein expression did not affect in vitro functionality of gene-modified T cells, warranting further evaluation of their anti-tumor activity in animal models. Overall, our study describes the ZF-Rs as an innovative epigenetic technology for engineered T cell therapies.

P213

Development of an *ex vivo* gene therapy for infantile gm1-gangliosidosis

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GM1-gangliosidosis (GM1, OMIM #230500) is a rare, recessive Lysosomal Storage Disorder (LSD) caused by mutations in the *GLB1* gene encoding the β-galactosidase (β-gal) enzyme. The enzymatic deficiency induces several mechanisms of cell and organ damage resulting in a severe, progressive neurodegeneration and premature death of the patients. LSDs require a therapy providing a prompt and robust enzyme delivery both to the central nervous system, and the periphery to prevent secondary pathological manifestation. *Ex vivo* Gene therapy (GT), i.e. the

autologous transplantation of Hematopoietic Stem/Progenitor Cell (HSPCs) genetically-corrected by lentiviral gene transfer, may represent a valuable therapeutic option for GM1, as proven in other clinical studies for LSDs.

To this aim, we developed two alternative therapeutic lentiviral vectors (LVs) expressing either a codon-optimized sequence of the human *GLB1* gene, or the murine *Glb1* cDNA. In agreement with previously published results, our data suggest that the murine β-gal is ~ 30-folds more active than the human enzyme in human cells, supporting the rational for its usage as putative therapeutic enzyme in a GT approach.

In GM1-patient primary fibroblasts, transduction with the therapeutic LVs determined a dose-dependent and supra-physiological enzyme expression. The transgenic enzyme was correctly secreted and uptake by target cells. LV transduction determined a stable metabolic correction of GM1 cells, proven by the rescue of storage material as early as two weeks post-transduction with an average vector copy number (VCN) of ≥ 3.

We are currently proceeding with the *in vivo* evaluation of our *ex vivo* GT strategy in a feasibility and efficacy study performed in *Glb1*$^{-/-}$ mice, animal model of the disease. GM1 mice show neurological symptoms by 18-20 weeks of age, and succumb to the disease by 40 weeks of age. The animal study included Busulfan-myeloablated 8-weeks old GM1 mice receiving *Glb1*$^{-/-}$ HSPCs transduced with a therapeutic or a control LV expressing GFP, or wild-type (wt) cells to mimic allogeneic HSC transplantation. Control groups included wt mice receiving mock-transduced cells, or untreated wt and GM1 mice. At 10 weeks post-transplant, a significant reconstitution of the enzymatic activity was observed in the peripheral blood mononuclear cells of GT-treated mice comparable to the wt level with the muβ-gal LV (VCN ~ 1.5) or to 50% of the wt level with the huβ-gal LV (VCN ~ 6). This biochemical correction was coupled to a significant amelioration of the neuromuscular phenotype evaluated at the rotarod, hanging and balance beam tests, and to a longer survival of the treated GM1 mice up to the end of the study (1 year of age). Notably, the use of the muβ-gal LV for HSC gene transfer resulted in a higher enzymatic activity and a comparable phenotypic correction as the huβ-gal LV but at a ~10-fold lower VCN, confirming a higher therapeutic potential of the murine enzyme.

In summary, our GT strategy has the potential to significantly ameliorate the phenotype of GM1 mice, with the murine β-gal LV showing an advantageous dose-effect profile, and may represent a novel opportunity for the treatment of GM1 patients.

P214

Investigating the biological properties of circulating hematopoietic stem/progenitor cells in paediatric subjects as novel source for gene therapy

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Despite most Hematopoietic Stem/Progenitor Cells (HSPC) reside within the bone marrow (BM) under physiological conditions, small numbers of circulating HSPC (cHSPC) are found in peripheral blood (PB). Data generated by our group showed that adult-derived cHSPC present similar *in vitro* multi-differentiation potential and *in vivo* BM-homing capability as compared to the BM-resident counterpart. Of note, during the first years of life the absolute counts of cHSPC are significantly higher than adults (3,7-fold), but little is known about the functional properties and molecular features of paediatric cHSPC, as well as their possible exploitation as novel stem cell source. Indeed, conventional HSPC harvests through BM aspirates or PB mobilization still present technical difficulties in very young individuals. Thus, the aim of this project is to comprehensively characterize the biological properties of the circulating HSPC compartment in paediatric individuals, and to assess the use of these cells as source for HSPC-based Gene Therapy (GT) strategies. By applying multi-parametric immunophenotyping to a cohort of 72 healthy donors (n = 45 paediatric subjects ranging from 0 to 10 years and n = 27 adults), we observed enriched levels of the primitive $CD38^-$ cHSPC fraction in paediatric individuals, with the preservation of their clonogenic potential. Furthermore, to analyse their functional properties, we performed a newly designed *in vitro* multi-lineage differentiation assay on single primitive $CD38^-$ HSPC subsets. Our data show that paediatric cHSPC display higher differentiation efficiency (60% in paediatric vs. 45% in adults) and increased multi-lineage hematopoietic production than adult cells. These data will be combined with transplantation in immunodeficient mice to measure the homing capability, hematopoietic reconstitution and long-term maintenance of paediatric cHSPC. Moreover, to study the molecular features of paediatric cHSPC, we combined phenotypic and transcriptome profiling at single-cell level on 29'275 BM-resident and 7'647 circulating HSPC isolated from 10 paediatric subjects. Finally, preliminary data indicate that paediatric cHSPC can be efficiently transduced with lentiviral vectors and expanded *ex vivo* exploiting state-of-the-art expansion protocol at comparable rate with respect to cord blood-derived HSPC. Overall, our project will generate fundamental information about the biology of paediatric cHSPC, paving the way for their clinical exploitation as stem cell source for *ex vivo* and *in vivo* HSPC-GT strategies.

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ISAnalytics: enhancing bioinformatics workflows in hematopoietic stem cell gene therapy

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Molecular monitoring of the clonal dynamics of hematopoietic reconstitution in Hematopoietic Stem and Progenitor Cell (HSPC) gene therapy is fundamental for assessing the long-term safety and efficacy of the treatment. This process involves high throughput sequencing, mapping, and genomic feature annotation of sequences flanking the integration site, which serves as a surrogate of clonal identity. For these analyses, robust and automated bioinformatics tools are strongly needed.

ISAnalytics, a novel open-source R package, has been developed to meet this need. It employs parallel processing and optimized data structures, facilitating efficient data analyses and interpretation of extensive clonal tracking data for large-scale longitudinal studies.

Various factors can impact the quality and noise level of input data, such as discrepancies due to sample preparation, sequencing depth, and PCR efficiency. ISAnalytics considers these variables

to recalibrate the results, ensuring the accuracy and reliability of the data analyses. The package provides several automated, fast, and reliable methods to address the consistency of the results.

Thanks to ISAnalytics, we observed that the number of Integration Sites (IS) retrieved from cell clones with defined numbers of IS was exceedingly high compared to the expected number or real IS present in each clone. Sequence analysis showed that the excessive number of IS retrieved was caused by phenomena such as index switching and primer jumping PCR.

In the case of index switching, the nucleotide barcode used to identify the specific sample is fused to the IS of other samples resulting in assignment of an IS to multiple samples. In the case of primer jumping PCR, during the elongation phase the newly synthesized strand detaches prematurely from the DNA template and anneals to other complementary sequences from other IS forming chimeric reads mapping in different portions of the genome, resulting in an inflation of the number of IS.

To solve or mitigate these issues, we explored a series of tools. We found that index switching occurs at a low frequency (from 0.1% to 2%, as reported by Illumina) when the barcodes used in the sequencing run are well differentiated and that false IS can be removed by addressing their significantly lower relative abundances compared to the parental IS.

On the other hand, the identification and removal of chimeric sequencing reads is much more complex as it involves the analysis of overrepresented sequence motifs present in the portions flanking the IS and distinguishing them from the parental sequence that triggered their generation during PCR amplification. Our proposed strategy is based on identification of motifs using the STREME algorithm.

In addition to the motif identification, IS abundance provides an indication for chimeras elimination, due to the fact that the true IS are several orders of magnitude more abundant with respect to chimeric reads.

In conclusion, ISAnalytics stands as a versatile tool that enhances the efficiency and adaptability of bioinformatics workflows in gene therapy, with a particular emphasis on data cleaning.

P216

Approaching optimal conditions for the treatment of Primary Hyperoxaluria Type 1 by *in vivo* lentiviral vector gene therapy

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Primary Hyperoxaluria Type 1 (PH1) is a rare autosomal recessive metabolic disorder caused by mutations in the *AGXT* gene. *AGXT* encodes the liver enzyme alanine glyoxylate aminotransferase.

PH1 patients suffer from oxalate overproduction, which can lead to end-stage renal disease and life-threatening oxalosis. Thirty percent patients show infantile onset. The only permanent curative treatment is double liver and kidney transplantation. Lentiviral vector *in vivo* gene therapy has emerged as a promising strategy for liver monogenic diseases, especially for pediatric patients, taking advantage of its integrative capacity.

In previous studies we reported that *in vivo* injection of a lentiviral vector (LV) encoding an enhanced AGT protein into *Agxt1* KO mice ($2x10^{10}$ TU/kg) showed a partial pathological phenotype reversion. Furthermore, increasing LV dose didn't improve therapeutic efficacy. Since PH1 is a cell-autonomous disease, improvements in therapeutic efficacy are directly related to increases in transduction efficiency. LVs could induce an IFNα-mediated immune response when injected *in vivo*. We reported that a transient immunosuppressive treatment with dexamethasone tripled basal transduction efficiency. Here, adult *Agxt1* KO mice were intravenously injected with a hepatocyte-specific LV expressing an *AGXT* cDNA in combination or not with dexamethasone. Treated *Agxt1* KO mice showed a significant reduction in urinary oxalate concentration compared to mice treated with the LV alone, resulting in a significant improvement of the therapeutic effect.

Based on the integration ability of LV, we hypothesized that this strategy could be efficiently used in a pediatric context. Thus, we intravenously injected neonatal and 3-week-old C57BL/6 wild-type mice with a hepatocyte-specific reporter LV expressing eGFP. These animals were analyzed at different time points with a maximum follow-up of 3 months and showed a maintenance of transduction percentage over time. However, in mice analyzed 3 months after injection, we observed a rather high variability, with some of them losing the transduced cells. Interestingly, we observed a significant increase in transduction efficacy in three-week-old injected mice compared to adult mice with a comparable vector copy number (VCN). Consequently, three-week-old *Agxt1* KO mice were intravenously injected with a hepatocyte-specific LV expressing an *AGXT* cDNA and sacrificed six months later, providing a long follow-up study. We observed a maintenance of LV integrations and detectable transgene expression, suggesting that, in contrast to our observation with the reporter LV, there was no loss of therapeutic LV transduced cells during the growth of the animals. We observed a 29% reduction in urinary oxalate levels in LV-treated animals compared to untreated animals, achieving a partial reversion of the pathological phenotype.

Overall, we observed a significant improvement of the proposed strategy in dexamethasone-treated adult PH1 mice and a 6-month maintenance of the therapeutic effect in three-week-old-treated mice. These results bring *in vivo* LV gene therapy a potential option for PH1 patients.

P217

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 cyclosporine analogue, CycloVect, that mediates IFITM3 degradation and potently enhances LV transduction of HSPC. Mechanistic studies indicate that CycloVect disturbs natural IFITM3 recycling, rerouting it to be degraded in lysozymes. Proteomic experiments to confirm the specificity of CycloVect are underway. Thus, CycloVect works downstream from, and additively with, current gold standard transduction enhancers that promote LV attachment. CycloVect is not immunosuppressive because it does not target CypA. It represents a promising new agent to enhance and standardise HSPC modification outcomes as well as a valuable tool to probe IFITM3 biology and restriction mechanisms.

P218

Lentiviral vectors endogenized by human syncytins, enable in vitro and in vivo gene transfer in human B cells

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Human syncytin-1 (Syn1) and syncytin-2 (Syn2) are fusogenic glycoproteins found at high levels in placenta and expressed from endogenous retrovirus env sequences in the human genome. These fusogens were used to pseudotype recombinant lentiviral vectors (LV) to obtain particles containing only human components in their envelope. Such “endogenized” LV would be presumably less immunogenic than viral-pseudotyped LV, currently used. Syn1 or Syn2-LV particles were produced in HEK293T cells at similar physical titers than VSV-G-LV but were slightly larger in size. The in vitro infectivity of Syn1-LV or Syn2-LV was strongly enhanced by transduction additive like vectofusin-1 (VF1). In these conditions, Syn1-LV and Syn2-LV selectively transduced some cell lines and not others. In blood mononuclear cells, Syn1-LV or Syn2-LV transduced CD19+B lymphocytes and dendritic cells at high levels compared to CD3+ T cells (respectively 59%±23; 59%±18 versus 11%±19, n=6), correlating with the levels of expression of ASCT2 and MFSD2a which are respectively the receptors for Syn1 and Syn2. The results obtained in B cells were remarkable because non-activated quiescent B cells could be transduced efficiently with syncytin-LV. In addition, up to 90% of B cell transduction could be achieved in some experiments, although these results depended on high titers and on the culture medium used. Syncytin-pseudotyped LV transduced all subsets of primary human B cells including CD19+, CD27-, IgM+, IgD+ naive B cells and CD19+, CD27high plasmablasts. Transduced B cells could expand in response to IL-21 and CD40L activation. Intravenous injection of Syn1-LV to NSG previously engrafted with PBMCs, demonstrated the possibility to transduce human B cells in vivo. Althogether, these results provide the first evidence of functional interactions between syncytins and B cells leading to novel perspectives in immunology. They also provide new tools for gene delivery to primary human B cells and further evaluations of immunogenicity and in vivo application of Syn-LV are ongoing.

P219

MaxPax™ – a simplified, rev/RRE-independent lentiviral vector with more space for cargo, and sequence-upgraded polyA LTRs (SupA-LTR™) for improved transcriptional insulation.

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Third generation lentiviral vectors (3rd Gen LVs) have an excellent safety profile, and remain the go-to choice when delivering larger genetic payloads to dividing and non-dividing cells. The core features of 3rd Gen LV genomes are: independence from tat, the packaging sequence (ψ), the rev-response element (RRE) enabling production of unspliced genomic vRNA, the central polypurine tract (cppt), and the self-inactivating (SIN) LTR, which improves safety. The architecture 3rd Gen LVs has not changed appreciably over the last 25 years. As part of our TetraVecta™ next generation LV platform we've developed the MaxPax™ LV genome with ~1kb increased transgene capacity to service the demand for delivering ever larger payloads.

This is achieved through mutation of the major splice donor (MSD) site within the packaging sequence, and the utilisation of an optimised 'Vector-Intron' (VI) to replace the RRE entirely. The MSD mutation is present to ablate aberrant splicing from this site to internal transgene sequences that would otherwise lead to spliced vRNA, which can be passively incorporated into 3rd Gen LV final products. MSD-inactivation within 3rd Gen LVs leads to a substantial loss in vector titre but in the case of MaxPax™ this effect is avoided by use of the VI. The splicing-out of the VI during pre-vRNA biogenesis stabilises the vRNA and consequently does not contribute to overall vRNA length – an important factor in RNA pool steady-state and vRNA packaging efficiency. Thus, the MSD-inactivation mutation and VI form a type of molecular symbiosis; mutation of MSD means that it cannot splice to internal sequences (including the VI splice acceptor), and the action of the VI rescues the attenuation effect of MSD-inactivation. We found that this configuration also allows the deletion of the majority of gag sequence present within the packaging sequence, which contributes to the increase in capacity. Since MaxPax™ vRNA is 'treated' like a cellular transcript – and export is mediated via Tap/Nxf1 – other introns such as those of the transgene cassette (e.g. EF1a intron) will be spliced-out. The gene therapy field is focussed on development of shorter, more powerful promoters that are not dependent on the use of introns. Therefore, MaxPax™ is ideal for large or over-sized transgene payloads where minimal *cis*-acting elements are employed, and introns are not required.

Additionally, MaxPax™ LV genomes are paired with SupA-LTR™ technology, that harbours engineered sequences within the LTRs to provide bi-directional transcriptional insulation to the integrated LV cassette, and enhance transgene expression. Finally, MaxPax™ LVs are also superior to 3rd Gen LVs in their ability on-board the TRiP System™, our standard approach to suppressing transgene expression during LV production, which allows rescue in output titres for LV genomes encoding 'problematic' therapeutic transgenes.

Here we describe the development of MaxPax™ and provide examples of production of LVs encoding therapeutic genes.

P220

Dynamics of hepatocyte heterogeneity in post-natal liver growth impacts *in vivo* LV gene transfer and gene editing

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The liver is an attractive target organ for *in vivo* gene therapy. Importantly, adeno-associated viral (AAV) vectors have recently obtained marketing authorization for adult patients affected by hemophilia. However, their use in pediatric patients is challenged by their non-integrating nature and consequent dilution of the episomal genome upon cell proliferation during liver growth. Lentiviral vectors (LV) are a promising alternative since they integrate into the cell genome. We achieved targeted transgene expression into hepatocytes, and stable gene transfer in adult mice, dogs, and non-human primates by LV intravenous (i.v.) administration. Here, we investigated the impact of spatio-temporal dynamics of hepatocytes during post-natal growth on transduction efficiency and transgene maintenance over time. We observed that LV i.v. administration to young (newborn or 2-week-old) mice leads to a 4-fold higher LV-positive liver area compared to adults, with preferential transduction of the peri-central area in young-treated mice and peri-portal area in adult-treated mice. We then evaluated proliferation of LV-transduced and untransduced hepatocytes, by administration of EdU during growth. We observed that 20% of hepatocytes were EdU-positive in young mice, with no differences among transduced or untransduced hepatocytes, while proliferation was almost absent in adults, indicating that only a fraction of hepatocytes proliferates during post-natal growth. Moreover, we noticed higher concentration of proliferating hepatocytes in peri-central compared to peri-portal area, together with LV-transduced hepatocytes, and this led to a 2-fold increase in transgene-positive area during the first 2 weeks after administration, which then remained stable over time. Since homology-directed repair (HDR) is reported to occur preferentially in proliferating cells, we measured HDR-edited liver area overtime following genome editing with CRISPR/Cas9 system, that allows targeted integration of a donor construct in a specific genome locus. We administered i.v. two AAV vectors in newborn mice (post-natal day 1), encoding for Cas9 and gRNA, and donor DNA, respectively, to integrate a promoterless mCherry transgene in frame with the albumin gene, separated by a self-cleaving 2A peptide. We observed 2-3-fold more proliferating hepatocytes among edited compared to non-edited ones, resulting in a 2-fold increase in the edited area following post-natal growth. We then performed spatial transcriptomics analysis of livers of young and adult mice to analyze changes in transcriptome profile that could affect hepatocyte permissiveness to LV transduction. In young mice, hepatocytes showed a peri-portal transcriptional profile, regardless of their localization in the lobule, lacking a fully established metabolic zonation. Interestingly we identified the proteasome pathway as a possible cell-intrinsic restriction factor, since it was highly expressed in adult peri-central hepatocytes compared to young livers. Thus, we administered the proteasome inhibitor bortezomib to adult mice before LV and achieved 4-fold higher liver transduction compared to LV-only treated mice, and a switch to peri-central transduction bias, indicating that proteasome plays an important role in determining the efficiency and distribution of LV hepatocyte transduction. Overall, our results indicate that age at treatment impacts on LV transduction efficiency and its spatial distribution in the liver lobule. These studies will inform further development of liver-directed gene therapy towards application to pediatric patients.

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Lentiviral gene therapy with tagged IDS causes efficient and cell engraftment-independent correction of brain pathology in Mucopolysaccharidosis type II

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Mucopolysaccharidosis type II (OMIM 309900) is lysosomal storage disorder (LSD) caused by Iduronate 2-sulfatase (IDS) deficiency and accumulation of glycosaminoglycans, leading to severe progressive neurodegeneration in pediatric patients. As intravenously infused enzyme replacement therapy cannot cross the blood-brain barrier (BBB), it fails to treat the brain, highlighting the unmet medical need to develop alternative therapies. We have tested modified versions of Hematopoietic Stem and Progenitor Cell (HSPC)-mediated Lentiviral Gene Therapy (LVGT) using IDS-tagging in combination with the strong ubiquitous MND promoter to optimize efficacy in brain and to investigate its mechanism of action in a mouse model. We found that tagging of IDS strongly improved correction of brain heparan sulfate, as measured with mass spectrometry, neuroinflammation and lysosomal pathology, as measured with immunofluorescence of CD68, GFAP and LAMP1, at low, clinically-relevant vector copy numbers. HSPC-derived cells engrafted in brain areas with efficiencies that were highest in perivascular areas (50 % of total), lower in choroid plexus and meninges(25 % of total), and lowest in parenchyma (10 % of total). The efficacy of correction of brain pathology was independent of the number of brain-engrafted HSPC-derived cells, but correlated with plasma levels of tagged IDS protein. These results indicate that tagged versions of IDS can outperform untagged IDS in HSPC-LVGT for the correction of brain pathology in MPS II, and they imply that the mechanisms involved include both cell-mediated and protein-mediated passage across the BBB. These results identify novel improved options for clinical development of HSPC-LVGT for MPS II.

P222

Improving the efficiency of in vivo lentiviral gene transfer to hepatocytes, by targeting anti-viral pathways, and application to familial hypercholesterolemia

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Lentiviral vectors (LV) are attractive vehicles for *in vivo* liver gene therapy, representing a complementary strategy to adeno-associated viral (AAV) vectors. In contrast to AAV, LV integrate in the genome of target cells, potentially ensuring maintenance of the transgene during liver growth, avoiding its dilution upon cell division. We have previously shown stable gene transfer to

the liver in mice, dogs and non-human primates by a single intravenous (i.v.) LV administration. Liver directed LV gene therapy may be applied not only to non-cell-autonomous diseases (like plasma protein deficiencies) but also to cell-autonomous disorders (like familial hypercholesterolemia, FH, caused by mutations in low density lipoprotein receptor, LDLR). However, high quantity of highly purified LV would be required for clinical translation. Moreover, to successfully correct cell-autonomous diseases, maximizing the percentage of corrected hepatocytes, rather than enforcing overexpression of the transgene of interest, would be preferable. For this reason, it would be crucial to improve the efficiency of LV gene transfer into hepatocytes. To achieve this purpose, we focused on anti-viral pathways, targeting them before LV administration. We identified inhibition of interferon αβ receptor I (IFNARI) and of proteasome activity as transduction enhancers. Indeed, a single dose of either anti-IFNARI antibody or the proteasome inhibitor bortezomib before LV injection increased the circulating amounts of coagulation factor VIII or IX, used as reporters, of about 5-fold. Additionally, we identified the procedure of fasting the mice for 24 hours (known to increase hepatocyte gene transfer by AAV vectors) as a third transduction enhancer, inducing about 7-fold higher transgene output compared to controls. We tested the combination of the previously identified enhancers and found fasting + bortezomib to confer the highest advantage, with >11-fold higher transgene output, compared to controls (maintained long term, 9 months of follow-up), while bortezomib + anti-IFNARI antibody did not synergize. LDLR is reported to be the main entry route of vesicular stomatitis virus glycoprotein (VSV.G) pseudotyped LV. However, during fasting, liver LDLR expression was considerably reduced, still resulting in an increased gene transfer. For this reason, we considered that even *Ldlr-/-* mice, mouse model of FH, would be amenable to transduction by LDLR encoding VSV.G LV. We thus treated juvenile *Ldlr-/-* mice and obtained long-term LDL normalization and prevention of atherosclerosis. Since in this setting the percentage of corrected hepatocytes might be more crucial than transgene overexpression, we tested the basal alpha-1-antitrypsin (HAAT) hepatocyte specific promoter compared to the strong enhanced transthyretin (ET) promoter, used in the previous experiments. At matched LV doses, HAAT was ~20-fold less potent in a hepatocyte cell line. Interestingly, we found similar therapeutic efficacy of LV equipped with the different promoters at similar doses *in vivo*. Here we report a number of relatively simple and potentially clinically viable interventions that allow achieving substantially increased potency of *in vivo* LV gene transfer to hepatocytes. Interestingly, reduction of the LDLR in the liver increased rather than decreased LV gene transfer. We exploited this favorable feature to successfully treat FH by liver-directed LV gene therapy in the mouse model.

P223

Optimization of retrieval and quantification methods for vector integration sites

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Specialized PCR-based techniques combined with next generation sequencing (NGS) and bioinformatics analyses allow the retrieval and mapping of vector integration sites (IS) in the genome of transduced cells. Because IS are stable genetic marks, distinctive for each independently transduced cell and its progeny, their characterization in gene therapy (GT) applications allows to obtain safety and efficacy readouts of the treatment.

We developed our method for IS retrieval, Sonication Linker mediated- PCR (SLiM-PCR), in which vector/genome junctions are specifically PCR amplified from sheared genomic DNA and sequenced by short-reads NGS. The identification and quantification of IS and the downstream analyses are performed by dedicated software VISPA2 and ISAnalytics. The abundance of each IS is obtained by counting the number of amplified DNA fragments of different length (depending on the shear site position) containing the same IS, that will be proportional to the number of cells contributing to a specific clone, avoiding the biases introduced by PCR amplification.

We validated SLiM-PCR for quantitative lentiviral (LV) and γ-retroviral (γ-RV) vector IS retrieval, using DNA standards composed of monoclonal samples carrying a defined number of IS in known positions of the genome mixed to a polyclonal sample harboring a random IS distribution. The results obtained showed i) high efficiency in IS retrieval ii) high correlation between the number of retrieved and expected IS ($R2 \simeq 0.9$) iii) observed abundance values of the monoclonal samples' IS matching the expected (observed/expected=0.96±0.1) up to 0.1% LV/γ-RV genomes over the total.
SLiM-PCR is thus our reference assay for IS analysis and it is currently being applied on thousands of samples coming from more than 30 preclinical studies and 7 different GT clinical trials exploiting different vector types like γ-RV, LV, SleepingBeauty Transposons and adeno-associated viruses (AAV). For AAV, because the vector genome breakpoint at the integration site is random, we exploited SLiM-PCR to interrogate different regions of the vector genome.

SLiM-PCR has been fully automated with liquid handlers able to process hundreds of DNA samples per week in 96/384-well plates. We are currently working on the miniaturization of the reactions exploiting the Acoustic Droplet Ejection technology of the Beckman Labcyte 525, an instrument capable of moving nanoliters of liquids by acoustic waves. So far we could reduce reactions volumes by 10 times, consequently achieving cost reduction, higher sample throughput, and higher overall assay speed and reproducibility.

To improve the reliability of our method, we are working on the optimization of the PCR conditions to reduce the generation of PCR artifacts, mainly originated by IS occurring in repetitive regions of the genome, that may potentially lead to false IS identification and consequently alter clonal quantification.

Moreover, we are setting up IS retrieval technologies based on long read sequencing, that can produce reads of kilobases in length, that would improve IS mapping.
In conclusion, our results showed that SLiM-PCR is a reproducible, accurate and versatile method that allows to perform high-throughput IS analyses. Moreover, our optimization process and the implementation of long read sequencing could increase resolution and reliability of our whole IS analysis workflow.

P224

Split intein-based ATM delivery with lentiviral vectors

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Ataxia telangiectasia (A-T) is a rare monogenetic, autosomal-recessive, multi-organ disease caused by mutations in the *ATM* (Ataxia telangiectasia mutated) gene. The corresponding protein kinase ATM plays an important role in the DNA damage response and coordinates DNA repair, cell cycle arrest, genome stability and apoptosis. Loss of ATM kinase function leads to a multisystemic

disorder characterized by an immunodeficiency causing recurrent infections and lung damage, progressive cerebellar degeneration resulting in ataxia, a high predisposition to cancer, sensitivity to radiation, premature aging, endocrine abnormalities and dilated blood vessels (telangiectasia). Life expectancy is highly affected, mainly due to chronic pulmonary diseases and malignancies. Since allogenic hematopoietic stem cell (HSC) transplantation in mice and human showed a positive impact on disease outcome, a gene therapy approach with autologous HSCs is a promising concept. Due to the large coding sequence of Atm (9.2 kb), efficient packaging of retroviral particles and transduction of target cells (HSCs) remains challenging. To circumvent the packaging limit of retroviral vectors, a dual vector system expressing proteins linked to split inteins can be used. Split inteins are protein segments enabling splicing of two independently expressed protein halves leading to the formation of a full-length protein. This process is called protein trans-splicing and first attempts in the field of gene therapy have been made with adeno-associated viral (AAV) vectors, since their packaging capacity is limited to <5 kb.

We developed a split intein system for expression of Atm, thereby delivering Atm sequence halves separated into two lentiviral vectors. For detection of both transgene halves by flow cytometry, the 5'Atm-N-intein sequence was co-expressed with GFP (in 5'position of 5'Atm using a F2A protease cleavage site) and the C-intein-3'Atm sequence was co-expressed with BFP (in 3'position of the 3'Atm using a F2A protease cleavage site). After efficient double transduction of Atm-deficient murine fibroblasts (up to 90% GFP-BFP double positive cells with MOI of 5) with the dual lentiviral vector system, trans-splicing efficiency with *Rma* (*Rhodotermus marinus)* DnaB split inteins was 3.7-fold more effective compared to the *Npu* (*Nostoc punctiforme*) DnaE split inteins and thereby demonstrating the reconstitution of full-length Atm protein. Whereas cells transduced with the *Npu* split intein system showed 47% of wild-type Atm levels, cells transduced with the *Rma* split inteins showed 174% of wild-type Atm protein level suggesting a superior trans-splicing efficiency of the later one. Taken together, expression of ATM by the split intein approach appears feasible and opening new possibilities for developing gene therapy for A-T.

P225

Effects of SERINC and IFITM proteins on the transduction efficiency of gene therapy lentiviral vectors

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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 efficient to treat rare immune defects and also more recently for the treatment of non-solid tumours. Major improvements in strategies and viral vector design have been made that facilitate lentiviral vector usage. However, owing to their viral origin, current lentiviral vectors are particularly sensitive to several innate immune proteins, called restriction factors, that severely restrict the transduction efficiency of specific cell lineages, among which the human hematopoietic stem/progenitor CD34+ cells that are of particular clinical interest to treat immune disorders. Here, using overexpressing and CRISPR-inactivated model cell lines, we systematically investigate the effects of individual members of membrane bound cellular proteins from the SERINC and IFITM families on the infectious properties of lentiviral vectors pseudotyped with several glycoproteins of potential interest for clinical applications. The ability of known restriction factor inhibitors of viral origin to relieve the blockage on lentiviral vectors

transduction is also tested to determine if they could be used to bypass the antiviral cellular responses and increase transduction efficiency of clinically relevant cells.

P226

HDR-based gene correction by co-delivery of Cas9/sgRNA RNPs and donor sequence in 'all-in-one' lentivirus-derived nanoparticles

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Genome editing using CRISPR/Cas9 allows targeted restoration of disease-causing genes and is particularly promising for treatment of monogenic disorders. However, current standard delivery approaches do not comply with safe *in vivo* delivery supporting short-term DNA cleavage activity. By adapting the properties of lentiviruses to package proteins, we have developed a CRISPR/Cas9 delivery technology based on packaging of Cas9 protein complexed with single guide RNA (sgRNA) as ribonucleoprotein (RNP) complexes in lentivirus-derived nanoparticles (LVNPs) for efficient knockout of target genes. Here, we explored the capacity of LVNPs to co-deliver Cas9/sgRNA RNPs and an RNA donor molecule, the latter being converted to double-stranded DNA by reverse transcription, for editing of targeted genes by homology-directed repair (HDR). Such approach may be particularly useful for applications requiring insertion of larger DNA segments. In HeLa and K562-derived reporter lines, we observed targeted HDR-based knockin of the *mCherry* gene in more than 60% of the cells treated with such 'all-in-one' LVNPs. Notably, potent knockin was dependent of the DNA-PK inhibitor M3814, suggesting that restriction of repair by nonhomologous end-joining was required to compensate for a delay in the availability of DNA donor. Additionally, we used this approach to tag the *LMNA* gene with a full-length *eGFP* gene (38% in HeLa; 13% in K562) and to introduce the E6V *HBB* gene variant causing sickle-cell anemia (up to 35% efficiency in K562). Interestingly, under conditions supporting similar on-target indel formation in the *HBB* locus using either standard RNP nucleofection or LVNP-directed RNP delivery, cutting in a known off-target site was frequent in nucleofected cells (51%) and very limited in LVNP-treated cells (5%). This difference matched differences in the cellular Cas9 concentration after delivery, suggesting that the potency of on-target DNA cleavage using LVNPs was achieved with lower levels of Cas9 protein resulting in limited off-target activity. Altogether, our results provide proof-of-concept for the use of LVNPs as 'all-in-one' delivery agents ferrying complete HDR genome editing tool kits with minimal off-target effects.

P227

F/HN pseudotyped lentiviral vector-mediated transduction of non-human primates

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Objectives
We have developed a lentiviral vector pseudotyped with the Sendai virus F and HN envelope proteins (BI 3720931) for cystic fibrosis (CF) gene therapy. We have previously demonstrated restoration of CFTR function in CF-patient bronchial epithelial cell air–liquid interface cultures and intestinal organoids, as well as efficient and persistent in vivo transduction of murine airways. We have now assessed transduction efficiency and acute toxicology in non-human primates (NHPs).

Methods
Male cynomolgus monkeys received a single dose of aerosolised rSIV.F/HN vector expressing green fluorescent protein (GFP) (4.2e9 transduction units in ~2 ml) or vehicle only (n=3/group via an endotracheal tube) and were culled 7 days after gene transfer. Approximately 25% of the dose (mass median aerodynamic diameter 3.9 µm) was deposited in the lungs. Toxicology was assessed by histopathology, clinical pathology, cytokine levels and changes in body and organ weight 2 days after gene transfer. Transduction efficiency was quantified by GFP immunohistochemistry and vector-specific mRNA.

Results
There were no rSIV.F/HN-related clinical observations, mortality, changes in body or organ weight. Clinical pathology and cytokine analyses were unremarkable. A minimal mixed-cell centriacinar inflammation was observed in one of three animals. Airway epithelial cell transduction efficiency was 9–12% and levels of vector-specific mRNA were ~45x higher than endogenous CFTR mRNA levels.

Conclusions
This study confirms and extends our previous findings of rSIV.F/HN-based in vivo gene transfer in mice. Using a relatively low vector dose, NHPs demonstrated transduction efficiency in the range of values likely to relate to clinical benefit, without evidence of toxicity. Transduction efficiency and toxicology in animals treated with a higher dose is currently being analysed. These data, together with our previous murine data, support further progression towards the clinic for this vector.

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P228

Mammalian myogenic cell fusogens (Myomaker and Myomerger) on enveloped vectors specifically target skeletal muscle for gene delivery

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A major issue with gene and cell therapies is an ability to deliver material specifically to the tissue of interest. Since the skeletal muscle fusogens (Myomaker and Myomerger) function specifically in skeletal muscle, we tested if their inclusion on enveloped vectors improve targeting of skeletal muscle. We report that engineering of Myomaker and Myomerger on the membrane of enveloped vectors leads to specific transduction of skeletal muscle, even in the absence of any additional targeting moiety or viral fusogen. We demonstrate that locally and systemically injected vectors pseudotyped with the muscle fusogens can deliver mDystrophin to skeletal muscle of a mouse model of Duchenne muscular dystrophy and alleviate pathology. We propose that vectors pseudotyped with Myomaker and Myomerger have the potential to circumvent some enduring challenges associated with AAV gene therapy, which currently dominates gene-corrective strategies for the muscular dystrophies. AAV gene therapy has potential issues including pre-existing immunity, toxicity, off-target effects, dosing-limitations, and limited transgene persistence, indicating that alternative vectors need to be developed. Lentiviruses pseudotyped with Myomaker and Myomerger described here have desirable characteristics including: a) targeting of myogenic progenitors, which has the potential to offer a life-long reservoir of therapeutic material b) immune evasion thus the possibility for re-dosing c) a larger transgene packaging capacity. Overall, we establish a specialized class of delivery vehicles that are trophic for skeletal muscle and can be developed into therapeutic modalities for genetic and acquired skeletal muscle diseases.

P229

Lentiviral CAR-T backbone promotor configuration determines vector production yield and vector functionality by modulating viral genome production and packaging yields

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VIVEbiotech is a GMP CDMO fully specialized in manufacture of Lentivirus-based transfer technologies, covering project from proof-of-concept to commercial stages. Our *Customer Development Service* is in charge of giving support form very early stages to our customers, offering solutions to unlock cost-effectiveness, productivity and functionality of their manufacture projects before transferring them to our highly scalable manufacture platform. Among others, a very powerful optimization strategy employed in VIVEbiotech for titer improvement relies on the design of the therapeutic plasmid promotor configuration. While external promoter has been previously described as a key factor for LVV titer, the internal promoter driving the expression of the transgene is usually selected under a transcription-on targeting criterion. In this work, we report the data obtained along the promoter interaction study in a collection of CAR-expressing transfer plasmids showing how both promoters have a significant impact on production yield and vector's infectivity. Our data demonstrate that an optimized configuration of the transfer plasmid promoters ensures greater efficiency and cost-effectiveness of cell therapy projects. Moreover, we propose mechanisms of action by which very commonly used promoters in CAR-T projects, decrease viral RNA transcription in producer cells, affecting negatively the packaging yield, vector infectivity and LVV titers, and should therefore be avoided to gain significant LVV productivity.

P230

Integrase-endonuclease fusion protein lentivirus vectors for targeted transgene integration

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Lentivirus vectors (LVs) enable long-term transgene expression by integrating their cargo into the genome of the host cell, which makes LVs a valuable tool especially in gene augmentation applications. However, random transgene integration leaves LV gene therapy at risk of side-effects caused by insertional mutagenesis. We have developed LVs containing the I-PpoI endonuclease fused to the viral integrase (IN) to reduce these side-effects. These targeting molecules can be packaged into the same vector particle with the therapeutic transgene constructs. The IN-I-PpoI fusion proteins direct LV-mediated transgene integration to the I-PpoI recognition site in the 28S subunit of the *RNA45S* ribosomal RNA gene. We have shown that IN-I-PpoI-LVs direct transgene integration away from protein-encoding genes and oncogenes, and thus generate a safer integration site distribution. We show that the targeting efficiency can be further enhanced with the use of homologous arms (HA), with sequence homology to the target site, flanking the transgene. We found that the HA-transgene constructs can also be used to improve the durability of transgene expression, to mitigate the effects of the IN-modification. We have tested LVs

containing differently mutated endonucleases, and found that these results are dependent on the type of IN-I-PpoI used in combination with the HA-transgene. IN-I-PpoIs capable of creating double-strand breaks (DSBs) in the target DNA lead to highly efficient integration targeting in the *RNA45S* across all tested cell types, but fusion-protein LVs with reduced DSB-cleavage efficiency produce more durable transgene expression. Furthermore, high DSB-cleavage activity can lead to deleterious effects in the chromosomal integrity of cells transduced with the fusion-protein LVs. Our studies show that IN-I-PpoI-LVs can be safely used to induce long-lasting transgene expression, but as with genome editing techniques, careful analysis of treatment effects is required after DSB-mediated genome editing.

P231

A platform for the design of highly active cell-specific synthetic promoters for gene therapy

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The critical objective of gene therapy is to achieve expression of a therapeutic gene at the required level in the specific target tissue for the desired period. The primary determinant of gene expression is the enhancer-promoter region, and, therefore, choice of promoter is central to the therapeutic success of corrective gene therapy. In the post genomic era, synthetic biology will allow *de novo* design of regulatory circuits that offer new opportunities to clinically manipulate at will transgene expression. As a step towards that objective, we have developed a bioinformatics-assisted pipeline to build and screen novel enhancer-promoter regulatory elements that do not exist in nature but that reflect the operational and activity criteria specified by the researcher.

Using in-house developed computational strategies, transcriptomic data, epigenetic datasets, motif enrichment, open-source data, and specifically generated tissue-specific data, we have developed a strategy for synthetic promoter design generating barcoded libraries of combinatorial regulatory motifs. These libraries can be rapidly screened using flow cytometry and NGS-based massively parallel reporter assays to identify enhancer-promoter combinations presenting the desired activity criteria.

Here we test such library designs by screening for enhancer-promoter architectures that are active in T-cell lines, but which have little or no transcriptional activity in non-haematopoietic cell types. This strategy provided a panel of synthetic promoters with transcriptional activity varying over a 40-fold range. These enhancer-promoters were highly cell type-specific with patterns of expression ~10-100 times stronger in target cells relative to non-haematopoietic cell types. Moreover, deletion analysis of several of these promoters identified the sequences contributing to activity and further validated the bioinformatics algorithms used to generate the libraries. With further sequence optimisation, we increased the specific activity of one of the champion promoters threefold, while reducing its size to 255 bp, representing an 80% reduction in functional size, and retaining the transcriptional cell specificity. Our data indicates that rational promoter design combined with suitable screening campaigns and engineering strategies are valuable tools for improving the performance of viral vectors and the on-target safety of gene therapies.

P232

Genome-wide characterization of the integration profile of a CD8-targeted fusosome gene therapy for *in vivo* CAR T cell generation

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Our *in vivo* T cell-targeted fusosomes may offer key advantages over autologous CAR T therapies via directed delivery and integration of a CD19 CAR transgene into CD8 T cells. In *ex vivo* CAR T therapies, T cells are conventionally transduced with VSV-G pseudotyped lentiviral vectors ("VSVG-LV") that integrate and mediate CAR expression. Our CD8-targeted fusosome is a third-generation self-inactivating lentiviral vector pseudotyped with a CD8-targeting fusogen ("fusosome-LV"). Use of the same integration machinery in our fusosome-LV should yield a comparable lentiviral integration profile to VSVG-LV; however, given the potential genotoxic risk from lentiviral integrations, we transduced activated CD8 T cells with fusosome-LV or VSVG-LV in three donors to compare the integration site (IS) profiles. Integrations were assessed 4- and 14-days after transduction via vector copy number (< 3.1 copies per diploid genome) and Illumina sequencing (> 46,000 total IS with fusosome-LV per timepoint). Initial analysis identified a ~13kb region on chromosome 8 that was potentially enriched in a single donor transduced with fusosome-LV at day 4 compared to VSVG-LV (FDR < 0.1), although this finding was not observed in the other two donors at day 4 or in the same donor at day 14. Additional analysis showed no evidence of preferential integration of our fusosome-LV near or within 736 Cancer Gene Census genes when compared with our VSVG-LV data and four *ex vivo* CAR T trials; our analysis includes loci (e.g., *MECOM*, *LMO2*, *CCND2*) previously associated with adverse events in clinical trials that used integrating gammaretroviral vectors. Given that our CD8-targeted fusosome-LV yielded no recurrent evidence for preferential integration and comparable IS profiles to VSVG-LV used in CAR T trials, these findings support further clinical evaluation of our fusosome-LV for *in vivo* CAR T cell generation.

P233

Enzymatically amplified linear doggybone DNA (dbDNA) as a rapid and scalable solution to industrial lentiviral vector manufacturing

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Traditional bacterial fermentation techniques used to manufacture plasmid are time-consuming, expensive, and inherently unstable. The production of sufficient GMP grade material thus imposes a major bottleneck on industrial scale manufacturing of lentiviral vectors (LVV). Touchlight's linear doggybone DNA (dbDNA) is an enzymatically amplified DNA vector produced with exceptional speed through an *in vitro* dual enzyme process, enabling industrial scale manufacturing of GMP material in a fraction of the time required for plasmid. We have previously shown that dbDNA can

be used to produce functional LVV, however obtaining high LVV titres remained a challenge. Here, we aimed to demonstrate that dbDNA could be optimised for the manufacture of high titre LVV. We found that dbDNA displayed a unique transfection and expression profile in the context of LVV production, necessitating a reduction in DNA input relative to plasmid of 50 – 70% in order to maximise titres. Furthermore, we found that efficient 3' end processing of viral genomic RNA (vgRNA) derived from linear dbDNA transfer vectors required the addition of a strong 3' termination signal and downstream spacer sequence to enable efficient vgRNA packaging. Using these improved vector architectures along with optimised transfection conditions, we were able to produce a CAR19h28z LVV with equivalent infectious titres as achieved using plasmid, demonstrating that dbDNA technology can provide a highly effective solution to the plasmid bottleneck.

P234

Validation of analytical method for lentivirus physical concentration using Videodrop (ILM) following ICH guidelines

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The EMA guidelines for quality control of gene therapy medicinal products recommend controlling the particle number and size of viral vectors Drug Substance (DS), Drug Product (DP), and critical intermediates in the viral vector process. Videodrop is an innovative system based on Interferometric Light Microscopy (ILM) which measures nanoparticle concentration and size quickly and easily. It can be included in the quality control plan. For this purpose, analytical methods must be validated following ICH Q2 guidelines. This study proposes evaluating the linearity and precision of Videodrop by analyzing lentivirus samples from various stages of bioproduction. The validation study includes two different bioprocesses and more than 200 measurements. The results of this validation study show a very good precision with CV< 12%, and excellent linearity with R^2>0,96. Results are favorable and hold promise for GMP methods validation for gene therapy products. Moreover, the very limited number of parameters and ease of use allow a very high reproducibility allowing easy QC transfer.

P235

Comprehensive genetic analysis of lentiviral vector integrations in genetically modified T-cells

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Retroviral and lentiviral vectors are commonly used to genetically engineer T-cells due to their high rates of transduction and long-lasting stable transgene expression. Since these vectors permanently integrate into the host cell's genomic DNA, thorough genetic characterisation and

quality control of integration sites is essential to ensure the safety, efficacy, and stability of T-cell therapy products.

Here, we genetically characterised 5 reference T-cell lines with zero to four lentiviral vector integrations per cell (samples VCN0-4) provided by the NIST genome editing consortium. Through serial dilutions with non-transgenic cells, samples with three different proportions of the transgenic cells (0.1%, 1%, 100%) were produced. TLA, a NGS technology based on proximity ligation, was performed on these samples with primer sets specific to the vector sequence to generate broad sequencing coverage into the host genome for hypothesis-free integration site detection. A bioinformatic peak detection tool for automatic and unbiased detection of these coverage peaks across the whole genome was used to identify integration sites. Small or structural variants in the vector sequence or in the host genome around integration sites were also assessed in the NGS data set.

Based on the broad coverage surrounding the integration sites, the peak detection tool identified all ten integration sites down to 0.1%. The previously reported integration on chromosome 4, placed in a region with non-unique sequences could not be confirmed. Instead, supported by broad coverage across the integration locus, TLA could unambiguously place this integration site on chromosome 16. The coverage also indicated a 648 kb genomic deletion near the integration site on chromosome 14, whereas no genomic structural variants were observed near or at the other integration sites. The integration site detection on nucleotide level allowed assessment of potential insertional oncogenesis. Seven of the ten integration sites occurred in intragenic regions, but none of the affected genes were listed as cancer genes in the *NCG7.0 network of cancer genes*. Vector integrity analysis revealed a small sequence variant in the integrated vector sequence of three samples. Based on the variant frequency, these variants are only present in one of the integrated vector copies.

Altogether, the data obtained for the NIST reference samples indicate that TLA is a powerful technology for genetic quality control of clonal and heterogeneous T-cell populations. With high accuracy, it simultaneously addresses several key genetic characteristics that are recommended by regulatory bodies for assessing safety of cell and gene therapy products.

P236

The impact of CRISPR-Cas gene cassettes on the packaging efficiency and transduction titre of lentiviral vectors

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CRISPR-Cas technology has revolutionised the use of genome-editing in studies on diverse biological topics. Lentiviral vectors (LVs) have been widely utilised for the generation of transduced cells that durably express the Cas nuclease and guide RNA (gRNA), the key components of the CRISPR-Cas editing system. The LV delivery system allows sustained gene expression *in vitro* and *in vivo*, in both dividing and non-dividing cells. A major challenge for the use of LVs in the clinical setting is the requirement to obtain sufficiently high titres for *in vivo* applications. This work aimed to assess the impact of the CRISPR-Cas transgenes, which differ in size, nucleotide composition and RNA structure, on the production of LVs and their ability to transduce cells. LV particles were produced by co-transfection of the packaging plasmids

pSYNGP, pRSV-rev and pVSV-G, with an equimolar amount of LV-plasmid in Human Embryonic Kidney 293 T (HEK293T) cells. Two days post-transfection, the LV containing supernatant was collected, filtered and concentrated approximately 20 times. The impact of the transgene length on RNA packaging was determined by quantifying viral RNA levels via cDNA production and PCR. The effect of the LV transgene length on virion production was assessed by measuring the CA-p24 protein level before and after concentration of the LV particles with an in-house developed CA-p24 ELISA assay. The correlation between the vector titre and the size of the LV transgene was evaluated by transduction of the SupT1 T cell line with concentrated LV particles. Three days post-transduction, the LV-titre was ascertained by quantitating GFP expression using flow cytometry. This study demonstrates that the transfer efficiency inversely correlates to the CRISPR-Cas transgene size, thus confirming the general rule about RNA size restriction. Remarkably, the LV transduction efficiency measured on the SupT1 cell line dropped dramatically for RNA transcripts approaching 8 Kb, as a further increase of 1 Kb reduced the transduction efficiency by more than 5-fold. As the number of produced virus particles was not affected, the use of larger transgenes most probably caused the production of empty virions. Even though we observed a striking inverse correlation between gene transfer efficiency and the insert size, this likely will not represent a perfect correlation, as other properties of the packaged RNA may still exhibit a modulatory effect. The efficiency of packaging of differently sized RNA transgenes determines the transduction titre, and thus the delivery efficiency of the LV system. Other variable LV properties, such as nucleotide composition and RNA structure, did not correlate with the transduction titre.

P237

A unique approach to minimising lentivirus transgene expression in production cell lines

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When manufacturing lentivirus using HEK293 based transient transfection systems, high levels of transgene expression has long been known to occur. This high level of expression is seen even when the gene of interest is under the control of tissue specific promoters which are effectively silent in HEK293 cells. Transgene expression during lentivirus production is understood to be problematic for production titres as the transgene may exhibit some toxicity or compete in some way with the other viral proteins. It was recently demonstrated by Cordes *et al* (University of Freiburg) that CD-19 CAR transgene expression during lentivirus manufacture can have an impact on potency by directing the lentivirus to off-target cells expressing CD-19. This work was based on an observation that CAR-T cell treatment of a B-cell leukaemia patient was not successful due to unintentional targeting of residual malignant B-cells.

Transgene expression in the manufacturing cell line has been shown to be very effectively managed by the inclusion of a sequences that inhibit transcription through binding to accessory proteins such as bacterial tryptophan RNA-binding attenuation protein. However, this does require additional sequence to be introduced into the vector upstream of the transgene. It also requires the expression of these accessory bacterial proteins within the manufacturing cell line, which is likely to be carried into the packaged lentivirus and introduced into the target cell.

Here we demonstrate a novel approach that significantly reduces transgene expression within the manufacturing cell line through the modification of the promoter sequence upstream of the 5' LTR. This modification has been demonstrated to reduce GFP transgene expression by 50-100

fold. We also see improvements in the production of other problematic payloads, as well as the potential to increase lentivirus quality by reducing carry-over of membrane proteins such as CARs. Importantly, the vector genome sequence is unaltered, and there is no requirement for any additional accessory proteins.

P238

Benchmarking NGS Integration Site Analysis methods in support of long-term safety monitoring of gene therapy products

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FDA and EMA Guidance states the importance of longitudinal testing of gene products introduced into human subjects. Depending on the delivery mechanism, the therapeutic gene product may or may not integrate into the genome. Of particular interest are gene-product integrations near proto-oncogenes which might lead to malignancies. This guidance states that recipients of an integrating gene therapy modality should be tracked for 15 years, while those receiving a non-integrating therapy modality should be tracked for 5 years. Therefore, advanced analytical methods are needed to identify, quantify, and track integration events across the genome. Here, we provide a comprehensive evaluation of methods leveraging next-generation sequencing approaches for genome-wide analysis of lentiviral integration events. Our analysis employed well-characterized standards consisting of varying copy number and known integration sites.

The approaches can be bucketed into two major groups: PCR amplification and target capture. All methods detected true positives with strong correlation to theoretical integration site dosage levels down to 1% allele frequency. Comparatively, PCR amplification-based approaches have lower data requirement per sample suggesting higher sensitivity, greater molecular capture, and lower limit of detection compared with target enrichment-based approaches. Target enrichment-based approaches can afford the flexibility to capture the integrated vector, which is of interest for characterizing partial integration events. While all methodologies performed well in our study, the choice of assay (or assays) for testing will depend on numerous factors including but not limited to the viral vector system and construct and starting material availability.

P239

Using a cell receptor knock-out strategy to enable stable expression of cytotoxic envelope glycoproteins for lentiviral vector production

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Gene therapy changed the paradigm of modern medicine by providing treatment to previously unmet medical needs, ranging from cancer to monogenic diseases. The use of lentiviral vectors (LV) in gene therapy clinical trials and licensed products has been steadily increasing in recent years. Nonetheless, LV manufacture still faces several challenges, namely the low yields and poor scalability of current production systems, hindering the transition from clinical to large-scale production settings. In this context, establishing efficient and scalable production processes based on high-titer constitutive producer cells is paramount to sustain the increasing clinical demand of LV-based gene therapies.

This work describes a new approach to establish constitutive LV producer cells stably expressing GaLV$^{\Delta R}$, an engineered, high-titer envelope glycoprotein. GaLV$^{\Delta R}$ promotes a 5-fold increase in LV titers relative to the original glycoprotein. However, due its fusogenic properties, GaLV$^{\Delta R}$ expression leads to syncytia formation when expressed at cell surface. Using CRISPR-Cas9 gene editing tool, we created a producer cell host deleted in GaLV cell receptor (PiT-1, inorganic phosphate transporter-1), to overcome GaLV$^{\Delta R}$ syncytia-derived cytotoxicity. PiT-1 knock-out (KO) cells supported GaLV$^{\Delta R}$ stable expression without syncytia formation, while maintaining transient LV titers. However, PiT-1 deletion led to a reduction in cell growth rate. Rescue of function assays were conducted by complementing PiT-1 KO cells with PiT-1 or PiT-2, another inorganic phosphate transporter. PiT-1 complementation rescued the syncytia formation phenotype upon GaLV$^{\Delta R}$ transfection. It also allowed an increase in cell growth rate to the levels of the parental cell line. These results support a link between PiT-1 deletion and the resistance to syncytia formation as well as the reduction on cell growth rate. PiT-2 complementation allowed a similar increase in cell growth rate while maintaining their resistance to syncytia formation, suggesting a cause-relation between the reduction on cell growth and the possible alteration on cellular phosphate levels.

PiT-1 KO cells are currently being used to develop new constitutive LV producer cells. To that end, PiT-1 KO cells were stable transfected with the remaining components required for LV production. After selection, cells with the highest levels of LV transgene expression were isolated by fluorescence-activated cell sorting (FACS). The resulting cells are currently being characterized for their potential for constitutive continuous LV production.

This work demonstrates the feasibility of stably expressing fusogenic envelopes by abolishing the expression of the corresponding receptors in the producer cell host. It generated new cell substrates currently being explored to manufacture LV through constitutive producer cell lines.

P240

[h]InGeTox: A human *in vitro* lentiviral genotoxicity assessment platform.

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1: Brunel University London 2: Testavec 3: Protagene 4: University Heidelberg 5: University of Edingburgh 6: University College London 7: National Institute of Biological Standard and Controls 8: University of the Witswatersrand 9: NCT & DKFZ, Heidelberg 10: Imperial College London

Lentivirus vectors are effective for gene transfer to treat genetic disease and cancer, however insertional mutagenesis and genotoxicity are important concerns. Currently used cell lines and animal models to risk assess LV are often over sensitive with outreads not wholly reliable to human gene therapy. [h]InGeTox is a human *in vitro* platform using induced pluripotent stem cells differentiated to hepatocyte like derivatives for specific assays to measure LV genotoxic potential. [h]InGeTox was applied to LV in SIN and LTR configurations for insertion site selection profiling in cancer genes (CG) coupled to differential expression, tracking of IS representative of clonal outgrowth within bulk cell populations of cells, interactions resulting in vector and host fusion transcripts and changes in epigenetic profiles of CGs that cause differential gene expression. [h]InGeTox represents the first human based model that provides reliable measurement of LV side effects for safe vector design.

P241

Automated systems leading solution to increase LVV characterization capacity

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AGC Biologics is a leading global Contract Development and Manufacturing Organization (CDMO), providing world-class development and manufacture of mammalian and microbial-based therapeutic proteins, plasmid DNA, viral vectors and genetically engineered cells.

Lentiviral vectors (LVV) are efficient vehicles for gene delivery that play an important role for ATMPs. The efficiency of the production process and the performance of the final LVV is monitored through potency and purity methods. In AGC Biologics, the infectious and physical viral titers and the ELISA assays to determine residual Host Cell Proteins, total residual DNA and residual Benzonase have been automated to increase capacity while also allowing the flexibility to run different assays in parallel using the same sample aliquot. The automated stations are constituted of a core liquid handling, full integrated with all the devices, Sealers, Real Time PCR thermocyclers, plate wash and plate reader, incubators to correct perform the different steps of the assays, including standards, controls and samples dilutions, procedure steps, results analysis.

The methods have been validated according to ICH Q2 (R1) before being transferred into automation and comparability of each assay's automated and manual methods has been

demonstrated (p ≥ 0.09 with paired t-test analysis). Automated methods have been qualified with repeatability ≤ 20% and a reproducibility with manual execution system ≤ 22%.

The infectious viral titer method is the most complex and time-consuming method, where the automation has brought the greatest help in terms of number of samples analysed at the same time. The assay is based on the detection of proviral DNA copy number integrated into the cell genome. The selected reference cell line, deeply characterized and full representative of target primary cells, is transduced with serial dilutions of vectors and tested with a qPCR assay.

Regarding infectious titer, the automated solutions brought to 5-fold increase in number of samples daily tested, greatly reducing the timing of analysis and operator effort. The overall data sets generated with automated stations on different purification steps or final purified LVV are not significantly different (p = 0.61 with paired t-test analysis), with a repeatability ≤ 15% and a reproducibility with manual execution system ≤ 19%.

The Elisa assays for the determinations of residuals HCP and Residual Benzonase with the fluorometric assay for the determination of total residual DNA, internally optimized to be suitable for GMP use, can be performed in parallel using a single dedicated aliquot of sample. The results obtained are comparable to one obtained performing the procedure manually (p ≥ 0.22 with paired t-test analysis), repeatability ≤ 20% and a reproducibility with manual execution system ≤ 22%.

All the automated stations, compliance to CFR21 and qualified according to ICH Q2 (R1), allow to have a complete automatic and traceable flow, from sample vial to result, that makes the difference in laboratory capacity.

P242

Comparison of the transduction efficiency of T-cells using LVV produced from suspension- or adherent-based production systems

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1: Yposkesi, an SK pharmteco company

Gene transfer technology using lentiviral vectors (LVV) is one of the most promising therapeutic approaches for cancer immunotherapy (CAR-T or TCR cells). However, an important manufacturing challenge is the industrialization of lentiviral vector production, especially given the potentially variable quantity/volume requirements for these new applications. Therefore, we have recently launched a scalable lentiviral vector manufacturing process based on quadruple plasmid transient transfection of HEK293T cells grown in suspension in bioreactors in serum-free conditions (50L- 200L scale), called Lentisure™. This platform benefits from more than 10years of cumulative experience in LVV manufacturing at Yposkesi. Given potential concerns between the product quality and product performance of LVV produced either by adherent- or suspension-based production systems, we have performed a comparison of LVV produced from both production systems.

The cell culture harvest from the suspension-based production system was purified by ion-exchange chromatography and concentrated by tangential-flow filtration. The overall yield of manufactured lentiviral vector was higher than that obtained with an adherent process, for a

comparable titre. The final LVV was characterized for infectious viral titre (IG/mL), particle content (ng p24), and process related impurities (protein and DNA).

We then evaluated the efficiency of this new lentiviral vector in parallel to a vector produced by an adherent manufacturing process. Results showed a comparable transduction efficiency of Jurkat cells and activated T-cells between lentiviral vectors produced by both adherent and suspension manufacturing processes, with a good correlation between multiplicity of infection (MOI), vector copy number and protein expression. We also demonstrated similar behaviour of T-cells transduced with a lentiviral vector produced either by an adherent or the new suspension processes.

In conclusion, lentiviral vectors manufactured by our Lentisure™ suspension platform resulted in a larger quantity of infectious and physical LVV particles, with comparable product quality, including transduction efficiency in T-cells, to lentiviral vector produced by more conventional methods (adherent-based LVV production). These studies may help clinical programmes who wish to transition between production systems (suspension, adherent) based on their clinical needs and future patient demand.

P243

Single-cell lentiviral vector integration sites and clonal tracking assay for cell and gene therapy

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1: Mission Bio

Lentiviral vector (LV) has been widely adopted as an efficient vehicle to deliver transgenes into cells due to its long-term efficacy. However, the semi-random integration of LV has raised safety concerns due to its potential to trigger tumorigenesis during CAR-T therapy. To mitigate this issue, characterization of vector integration on clonal expansion after gene therapy has become a crucial practice to monitor the activity of retroviral vectors on in vivo selection of patient clones. Here, we developed a single-cell resolution, lentiviral vector integration site assay to survey the co-occurrence of specific integration sites with somatic genomic variants. Based on a set of LV transduced cell lines with known integration sites validated by orthogonal data, a targeted panel was designed to cover 5' and 3' ends of each integration site with predefined integration orientations. Samples include negative control and LV transduced cell lines with known vector copy numbers ranging from one to four copies. Using a titration experiment design, we demonstrate the capability to quantitatively detect individual cells harboring specific vector integration sites and longitudinal tracking of cell clones with different vector copy numbers. Furthermore, another set of 99-plex human genome amplicons are included to characterize somatic variants in the cell population of a sample. Taken together, a high throughput single-cell multi-omics platform enabled us to simultaneously identify somatic variants along with vector integration events in individual cells, providing both potential functional mutation identification and clonal tracking capabilities. The development of single-cell lentiviral vector integration sites and clonal tracking assay provides a unique opportunity to better study longitudinally CAR-T cell clonal expansion and lead to a more effective therapeutic agent.

P244

Downstream strategies for concentration and purification of LV Vectors from suspension cell-based systems.

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1: AGC Biologics 2: Orchard Therapeutics 3: Cawsam BioProcess Consulting ltd

Lentiviral vectors (LVV), produced with transient quadri-transfection in 293T cell line, are extensively used for genetic modification in gene therapy, and have already shown great promise, with several clinical trials demonstrating their efficacy in treating diseases. The demand for LVV is constantly rising to support advanced clinical phases and commercial applications.

In this regard suspension cells-based systems offer a great advantage in scalability *vs.* traditional adherent systems widely used so far, allowing to design very large scale processes (batch volumes up to thousands of litres) that can support the increasing demands, with the advantage, at the same time, of removing animal sourced material (FBS).

Suspension cells-based systems however pose a number of challenges to the downstream phase that need to be addressed in order to maintain high process performance and LV vector quality in terms of low level of impurities.

The presence of producer cells in the harvested medium and the overall residual contaminants profile mark a difference in comparison to classical adherent systems. Cells and cellular debris must be removed from the LV-containing supernatant while minimizing cell breakage to prevent the release of cellular proteins and DNA. Supernatants from suspension-based systems generally show higher amounts of residual contaminants, which need to be addressed during the downstream processing (DSP) steps.

A proprietary DSP process has been developed in AGC as part of a co-development effort in collaboration with Orchard Therapeutics for the purification and concentration of LVVs produced in stirred tanks bioreactors that allows for easy scalability and application at vastly different scales, with current ranges of 50 to 200L and planned scaleup to 1000 and 2000L.

Clarification of the bulk supernatant was extensively studied, in order to identify the most suitable solution to remove cells and cell debris, while optimizing LV recovery. Depth filtration and single use continuous centrifugation systems were investigated. Optimized tangential flow filtration and chromatographic steps are needed to reduce the very large bulk volumes and to remove the higher levels of residual contaminants characteristic of suspension cells-based systems. The downstream scheme was studied to maximize vector recovery while preserving infectivity, and different chromatographic resins and membranes were scouted to identify the best balance between LV recovery, purity and infectivity. The final sterilizing filtration step – always critical for LVV processes – was tailored to accommodate the larger amount of product coming from suspension processes.

These purification approaches were then formulated in a process scheme that can be applied to widely different scales, from a <10L scale down model (suitable for development or preliminary testing activities) to 50L, 200L and up to 2000L, identifying the appropriate equipment and to seamlessly move between scales.

Scaled-down, 50L and 200L processes have already been executed, obtaining infectious viral titers of 5E+08 – 2E+09 TU/mL for the final purified vector, with an average overall process recovery of 20%-30%. Results will be presented along with the definition of the process.

P245

Shortening lentivirus process development timelines using a templated approach

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The field of lentivirus GMP manufacturing is relatively new, with only a handful of existing therapies on the market today. However, the success of these life-saving therapies is potentiating the development of novel lentivirus-based therapies and supporting a growing need for lentivirus GMP manufacture. Further, the timelines for getting regulatory-approved novel therapies to the clinic are compressed due to factors such as patient outlook and the competition. There is a need in the industry to simplify the path to rapid, robust, and compliant manufacture of lentivirus-based therapeutics. To address the key drivers of lentivirus manufacturing, we developed a vendor-agnostic, standard manufacturing template containing beginning-to-end guidelines to ensure that the process meets upstream and downstream performance metrics, raw materials meet compliance requirements, and timelines are predictable. Here we show a case study using this lentivirus manufacturing template for a model therapeutic. Using DOE-based approaches, we show rapid optimization of upstream bioprocessing and achieved harvest titers of >5x108TU/ml. The template permitted rapid and efficient scale-up to 50L scale and generation of high titer material for purification. Using pre-determined template parameters, we were able to obtain efficient recovery of the product with limited iterations. Step recoveries of each step in the downstream process will be shown. The collective reduction of process development time using this lentivirus template containing established protocols and compliant raw materials enables rapid manufacture and delivery of life-saving therapies to patients.

P246

Stable lentiviral vector production cell lines yield LVV titres comparable to the four-plasmid process

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Current lentiviral vector (LVV) manufacturing approaches primarily use fully transient and adherent processes, which present challenges of robustness, scalability, and high cost-of-goods. To address these, we have developed suspension cell lines for semi-stable and stable LVV manufacture. We first developed an LVV packaging cell line by random integration of

Tet-regulated VSV-G and Gag-Pol, and constitutive Rev (codon optimised and high homology regions of DNA deleted to decrease risk of recombination) into clonal WXATUS0028 suspension HEK 293 cell line, followed by clonal isolation of the top performing clone – LVPack13-14. We then developed an LVV producer cell line by transposon-facilitated integration of the LVV genome into the top packaging cell line clone, followed by clonal isolation and performance screening. When GFP was encoded as the GOI, both the packaging and producer cell lines consistently produced 1.5-3.0 $\times 10^8$ TU/mL for 27 passages without antibiotic selection. Copy number analysis by ddPCR for both packaging and producer cell lines showed no loss of gene copy number during this time. Furthermore, we trialled production of LVV encoding several different therapeutically relevant transgenes, with sizes ranging from 0.05 to 4.4 kb. All packaging/producer cell lines produced comparable or higher LVV titres than the four-plasmid transfection-based production method. This minimal effect on LVV production we observed when encoding various therapeutically relevant transgenes indicates the customisability of our technology. Scale-up vector production and process development with the packaging cell line are ongoing, with the master cell bank (MCB) being generated for GMP-standard vector manufacturing. In summary, our packaging/producer cell lines produce LVV at titres comparable to the industry standard four-plasmid method, enabling a smooth transition to a process with reduced manufacturing costs and variability, and improving patient access to LVV-based cell and gene therapies.

P247

Expediting lentiviral vector development with automated bioreactors and design of experiments

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Background & Aim:
Development of new Lentiviral Vector (LV) bioprocesses is often slow due to the difficulty in establishing optimal values for critical process parameters and the large time requirement to run analytical assays. Adding to the development time is extended work required with external manufacturing partners. The ability to make accurate, data-driven product estimations can greatly speed up development timelines.

Methods, Results & Conclusion:
LVs were produced in HEK293 cells transiently transfected in a 24-way Ambr®15. Infectious titers were calculated by LV transduction on H9 cells measured via flow cytometry. Experiments made use of DOE methodology in MODDE® to design, optimize, and predict bioreactor and process parameters, with results validated in the BioSTAT STR®.

The upstream process was developed in 3 phases. The first phase focused on cell line selection, TFX reagent selection and initial process parameters. The second phase focused on the LV production parameters and TFX optimization (PEI:DNA ratio/DNA plasmid ratio/Plasmids selection). The third phase focused on the target TFX VCD, the feeding strategy and a 50L scale-up golden batch.

The application of design of experiments (DOE) in combination with automated multi-way bioreactors with Ambr® 15 together with a variety of advanced tools and techniques allowed for rapid and efficient experimentation to identify ideal operating conditions that were able to be

directly scaled to a 50 L Biostat STR®. This project design allowed for rapid deployment of the process for GMP process operations.

P248

Precise measurement of vector copy number and transduction efficiency at single-cell resolution for cell and gene therapy development

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Cell and gene therapies are transformative solutions for patients with inherited and acquired diseases for which existing interventions are ineffective. The therapies target diseases at the cell level and modify or replace genes of specific sets of cells to alter their functionality. Such therapies usually rely on viral transduction or non-viral vector gene transfer techniques to introduce transgenes into host cells. The accurate measurement of gene transfer is critical to the development of therapeutic agents, which directly contributes to their safety and clinical efficacy. Currently, methods for the quantitative measurement of gene transfer lack the resolution and representation to truly reflect sample composition and either report a population bulk average or require labor intensive and time-consuming clonal isolation which can take weeks. Here, using a microfluidic, emulsion based single-cell DNA sequencing platform and a highly multiplexed amplicon panel targeting both vector sequence and human genome, we demonstrate a novel methodology that provides precise and accurate measurements of vector copy number (VCN) and transduction efficiency at single-cell resolution for cell and gene therapy development. A set of well characterized clonal cell lines with a range of known vector copy numbers validated by orthogonal methods was used for assay development. We show that single-cell DNA sequencing identifies transduced versus non-transduced cells with exceptional sensitivity (99.9%) and specificity (99.6%) with high precision. In the same assay output, populational VCN can be measured with high correlation (R squared value 0.99) compared to orthogonal ddPCR values. Due to the simultaneous measurement of transduction % and populational VCN, the assay also provides average VCN per transduced cells from the same data set. Finally, besides average VCN, the distribution of VCN at single-cell resolution can be characterized. The single-cell level vector copy number and transduction assay provides exceptional resolution and valuable insights for those developing more effective and safe therapeutic agents.

P249

Leveraging the potential of lentiviruses, mRNA, and LNP- coated RNA antibody conjugates in accelerating cell therapy programs

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1: Azenta Life Sciences

Cell therapy has emerged as a promising approach for the treatment of various diseases, offering potential solutions where traditional therapeutic methods have fallen short. Recombinant lentiviruses are the most broadly used viral vectors for efficient gene delivery into mammalian cells as they can stably integrate the gene of interest into the host genome. Their ability to efficiently deliver genetic material to a wide range of cell types enables the engineering of cells to exhibit desired functionalities or to correct genetic abnormalities. This approach has been widely used in CAR-T cell-based therapy programs. However, this approach may not suitable for patients with solid tumors or cases with low T cell counts.

In parallel, mRNA therapy has gained a substantial attention as a promising platform for delivering therapeutic molecules into cells. However, one limiting factor of this approach is an efficient *in vivo* delivery to the targeted tissues/cells. The advent of Lipid Nanoparticle (LNP)-coated mRNA, conjugated with an antibody specific to surface markers expressed on target cells has overcome this challenge, providing a safe and efficient means of delivering mRNA to target cell types. LNP-coated mRNA formulations can be customized to encapsulate specific mRNA sequences, allowing precise control over protein expression and cellular responses.

Here were offer a streamlined, and end-to-end workflow of from gene synthesis to lentivirus production with various solutions to tackle specific challenges, maximizing the success rate of cell transduction and cell line engineering. We also present Azenta Life Sciences' RNA synthesis capability; mRNA and Circular RNA (circRNA) that involves a rapid and accurate *de novo* synthesis and verification of template DNA, including complex polyA regions, to deliver accurate, high quality research grade mRNA in both linear and circular forms. This optimized synthetic workflow eliminates current limitations in quality, scalability, and speed of *in vitro* transcription (IVT), maximizing potential for success of mRNAs in downstream applications. Considering the LNP-mRNA antibody conjugate approaches, Azenta Life Sciences' Recombinant Antibody Production service provides a robust and streamlined process serving as a valuable tool for a targeted cell therapy program.

In conclusion, Azenta Life Sciences' synthetics services provide a full end-to-end spectrum of reagents that help accelerate cell therapy development.

P250

A rapid and novel ddPCR based RCL assay for use with fusosomes.

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1: Sana Biotechnology

Sana is developing a novel fusogen platform utilizing retargeted viral fusogens pseudotyped on lentiviral vectors called fusosomes, for *in vivo* gene delivery. Production of the fusosome utilizes standard third generation self-inactivating and split genome plasmids to minimize the risk of forming a replication competent lentivirus (RCL). Although these designs make RCL implausible, current FDA guidelines include testing both the gene therapy vector and producer cells to rule out RCL formation. Traditional RCL assays utilize the C8166 cell line and HIV-1 as a positive control with readouts based on p24 protein, qPCR, or PERT to assess for the outgrowth of replication competent particles. Because of the low sensitivity of the end point readouts, these RCL assays can take 50-86 days and require significant amounts of vector material to complete. Furthermore, use of HIV-1 as a positive control introduces biosafety level and handling challenges. Although the

traditional C8166 cells are permissive to VSV-G pseudotyped lentiviral vectors that are commonly used in *ex vivo* cell therapy, these cells may not be permissive to novel vectors being developed for *in vivo* use with selective tropism, including retargeted fusosomes. Therefore, we developed a novel RCL assay that utilizes a permissible suspension cell line for amplification with ddPCR endpoint using 4070A-MLV as a positive control. Importantly, this RCL assay can be applied to other targeted viral vector gene deliveries, while decreasing the assay time from 56-86 days down to 12-18 days utilizing a sensitive readout.

P251

Metabolite-based cell density estimation and harvest strategy evaluation for scalable and reproducible Lentivirus production with HEK293T cells in structured fixed-bed bioreactors

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1: UCL

Lentiviral vectors (LV) have been proven to be efficient gene delivery vehicles in both dividing and non-dividing cells. The traditional method of culturing adherent HEK293T cells for LV production is limited in scalability and reproducibility, leading to increased volume, manual operations, and high costs. Suspension processes in stirred tank bioreactors offer an alternative solution, however, transient transfection for LV production on STRs can increase the process volume, requires rapid removal of the product from the bioreactor due to the low stability and the storage conditions, and entails implementation of perfusion processes, which requires cell retention devices such as alternating tangential flow (ATF) filtration. All combined with the required dose size and quality of vectors for the gene therapies introduce further challenges in industrial-scale manufacturing for LV production.

Fixed-bed (FB) bioreactors offer promising features, such as a low footprint design and a cost-effective structure to overcome capacity, process volume, and perfusion implementation challenges for large-scale virus manufacturing, compared to STR and traditional adherence-based technologies. The aim of this study is to observe the productivity during the process transfer of LV production with a HEK293T cell line from a first-generation packed-bed (PB) bioreactor to next-generation novel structured bioreactors, outline a non-invasive method for accurate estimation of the cell numbers in a FB bioreactor using metabolite measurements as a complementary approach to direct FB sampling via sample carriers (SCs), and test two different harvest strategies to evaluate the LV production efficiency.

The results showed that the TU to p24 ratio was about 3 times higher in the scale-X hydro bioreactor compared to the PB bioreactor (Figure 1) These results along with a number of functional viral particles that was 10 times higher (Figure 2) suggest a higher transfection efficiency in the scale-X bioreactors. Additionally, the metabolite-based cell density estimation method is demonstrated to be easily applicable, non-invasive, and capable of producing accurate results, that can be used for cell monitoring and estimation of transfection time via measurement of lactate and glucose concentrations. Lastly, it is observed in the harvest strategies that production in batch mode had a 12.7% lower total yield of viral particles, and only half of the medium volume was used during production (~5.8 L) compared to production in continuous perfusion, which shows that it is a more cost-effective strategy. On the other hand, LV production in perfusion required more medium but fewer manual operations and allowed for continuous

removal and cooling of the harvest, which is advantageous for virus stability as well as for large-scale production.

Figure 1. The TU/p24 ratio for Lentivirus yields in pooled harvests in scale-X hydro bioreactor compared to a PB bioreactor

Figure 2. Lentivirus yields in pooled harvests in scale-X hydro bioreactor and in the PB bioreactor in % TU total pool (FACS) normalized to PB lentivirus yield

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P252

Abolishing retro-transduction of producer cells in lentiviral vector manufacturing

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Transduction of producer cells during lentiviral vector (LVV) production causes the loss of 70-90% of viable particles. This process is called retro-transduction and it is a consequence of the interaction between the LVV envelope protein, VSV-G, and the LDL receptor located on the producer cell membrane, allowing lentiviral infection. Avoiding retro-transduction in LVV manufacturing is crucial to improve net production and, therefore, efficiency of the production process. Here, we describe a method for quantifying transduction of producer cells and how this can be reduced using three different strategies based on the interaction between VSV-G pseudotyped LVV and the LDL-R.

P253

Lentiviral vector manufacturing for CAR delivery, a successful and reproducible continuum process from discovery to the clinical applications

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The need for Advanced Therapy Medicinal Products (ATMP) has exploded, especially for CAR-T and CAR-NK treatments. Providing highly purified lentiviral vectors (LV) as starting material for *ex vivo* clinical trials, from Discovery to Clinical phases, is a success factor for the development of cell and gene therapy products. Flash Therapeutics is a French biotech company positioned as a global CDMO expert in the manufacturing of lentiviral vectors used for gene addition or gene editing therapeutic approaches. Since 2005, more than 9,000 custom batches have been produced, including 50 large scale (30 to 200mL at about 1E9 IG/mL). Our proprietary lentiviral platform includes both regular integrative lentiviral vectors (iLV) and our proprietary non-integrative LentiFlash® technology. Through a continuum from research to clinic, we offer high quality vector manufacturing and personalized support. Ensuring a continuum from discovery to clinical

applications requires to successfully shift between LV production scales while maintaining process performance attributes and critical quality attributes. Flash Therapeutics conducted extensive data analysis through different steps of the GMP continuum and demonstrated a successful scale-up of LV manufacturing based on productivity and quality attributes such as infectivity ratio or process-related impurities content. A detailed analytical characterization of the product was established with GMP QCs and led to equivalent product specifications across different process steps, either considering integrated genome (IG/mL) or physical particles (PP). PP/IG ratios were low, ensuring high infectivity. This successful and reproducible continuum can include 4 bio-production steps from small scale research to large scale GMP. The success of clinical studies using ATMPs requires several intermediate stages of *in vitro* development involving a constant quality of lentiviral vector manufacturing. Here, we describe a successful and reproducible continuum process for LV manufacturing, leading to highly reliable process yield & quality attributes to generate LV batches to be used for various immune cells engineering that will be used for phases I/II clinical studies.Our patented production and purification process guarantees the same specifications from discovery to clinical grade, which finally ensures the success of your clinical study. The methodology allows an efficient gene transfer even into delicate or hard-to-transduce cells, while preserving their viability and phenotypic profile. CAR cells can thus be produced using MOI <5, while ensuring a high level of expression, with a reduced viral copy number (VCN).

P254

Finding the right path for Cell and Gene therapy analytics (and more)

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Cell and Gene Therapies (CGTs) are based on "live" drugs typically composed of cells, *ex-vivo* genetically modified cells, or genes directly transferred *in-vivo* by carriers or vectors, which are often customized for individual patients or small patient niches. Since 2009, when the first Advanced Therapy Medicinal Product (ATMP) was commercially approved in the EU, these treatments have shown brilliant results and, in particular, immunotherapies (e.g. CAR-T) and gene therapies for monogenic conditions have demonstrated remarkable outcomes.

Despite the intrinsic complexity poses specific challenges in their development, manufacturing, and quality control, Lenti Viral Vectors (LVVs) emerged as central tools for efficient gene modification, primarily in *ex-vivo* applications, leading to the successful commercialization of gene-modified cells for rare diseases and CAR-T for blood malignancies. Recently, there has been a growing interest in also utilizing LVVs for *in-vivo* applications, as an alternative to Adeno Associated Vectors (AAVs) and other vectors, possibly further emphasizing the importance of robust and rigorous analytical testing to ensure their safety and effectiveness.

Eurofins, a well-known first-class BioPharmaceutical outsourcing services partner, has a strong presence, among other, in the CGTs segment. Here we will present the ongoing efforts of the Biopharma Product Testing (BPT) division to establish analytical platforms to characterize, monitor and release LVVs, as well as LVV gene-edited cells. The primary objective is to ensure the safety of these products, by developing reliable and fit-for-the-purpose analytical solutions that could not only accelerate the manufacturing and release of LVV-based therapies, but also reduce costs and conserve valuable resources. Furthermore, the insights gained from this exercise can be

extrapolated to establish similar analytical platforms for other critical parameters, such as identity, purity, and potency, ensuring the overall safety and efficacy of cell and gene therapies.

The proposed platforms align with Eurofins' commitment to promote the sustainability and affordability of CGTs, which are central topics for the industry. Going beyond BPT activities, we will explore the identified opportunities of industrialization and innovation to establish the foundation for CGTs development according to "the Eurofins way" approach, driven by our testing-for-life philosophy.

P255

Detailed study of the biodistribution of Nipah virus pseudotyped lentiviral vector in healthy mice

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Lentiviral vectors (LVs) are beneficial as gene therapy (GT) vectors because they promote long-term expression, have broad tropism, can accommodate large transgenes, and are mildly immunogenic. LVs are usually pseudotyped with vesicular stomatitis virus glycoprotein (VSV-G) which has low-density lipoprotein receptor (LDLR) for cellular entry. Systemic delivery of VSV-G LVs in mice shows that the vector has broad tropism, such as for bone marrow, liver, spleen, heart, bladder, testis, lung, brain, and more tissues.

LVs can be pseudotyped with the entry proteins of many viruses, including the Nipah virus (NiV) which belongs to the family of paramyxoviruses. Ephrin B2 is the natural cellular entry protein for NiVs. It is expressed in most human tissues at medium levels and at high levels in the heart and testes. NiV tropism is obtained by pseudotyping LVs with truncated NiV glyco- (G) and fusion (F) proteins. Additionally, NiV-derived pseudotypes have helped in achieving transductional targeting of LVs, in applications such as the in vivo generation of tumor-eliminating immune cells. The broad tropism of VSV-G reduces its viability for specific gene therapy and thus there is a need to study different pseudotyped LVs capable of transducing desired organs. We are carrying out a detailed study to characterize the biodistribution of the NiV-LVs in healthy mice. The characterization will be based on bioluminescence imaging, analysis of transgene expression, and vector copy numbers in different tissues. As a result of our *in vitro* and *in vivo* testing, we present an analysis of the NiV LVs' biodistribution in mice in comparison to the regularly used VSV-G LVs. Our results can help in deciding the utility of NiV LVs in applications where transduction of tissues other than the liver is desired.

P256

The viral mosaic- Different aspects of lentivirus production and how to tackle them

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Lentiviral vectors (LVs) have become an increasingly relevant tool for gene and cell therapy applications. Their ability to transduce dividing and non-dividing cells and to stably integrate their transgene into the host cell genome has revolutionized possible treatment options in the field of immuno-oncology, like CAR-T cell therapy. However, as the demand increases, efficient production processes to achieve high quality LV yields are more important than ever. One important factor for an effective LV production process is the transfection protocol, as LV production still mainly relies on a transient transfection approach. Therefore, we investigated different transfection strategies, demonstrating the positive effect of fresh medium and cell splitting on LV production. To further increase LV yield, the influence of various cell culture supplements that we believe are critical for cell membrane synthesis and therefore LV functionality were evaluated. Among them were cholesterol and other lipid supplements, which were tested at different concentrations and addition times. While the Lipid Mixture 1 (Sigma), SyntheCol NS0 (Sigma) and the Chemically Defined Lipid Concentrate (Gibco) showed no notable effect on the infectious titre, the Cholesterol Lipid Concentrate (Gibco) had a critical negative effect with an up to 45-fold decrease in titre. Addition of this supplement during pre-culture showed an even stronger negative effect, which may result from hindering the transfection process. In spite of this, we have identified an in-house solubilised cholesterol powder (Sigma) that has a significant positive effect on the infectious titre. In another study, we aimed to increase viral titre by adding numerous enhancing compounds after transfection, such as HDAC inhibitors, achieving an increase in functional titre of up to 2.5-fold. As LVs are known to be fragile vectors, storage conditions for their shelf life are also important factors to consider for LV production. Hence, we conducted a stability study to investigate the stability of LVs at -80 °C, -20 °C and +4 °C. Our results demonstrate that LVs are stable not only at -80 °C, but also at -20 °C for at least six months. This work shows how several steps during LV production can be tackled to help overcome current limitations and therefore making LV-dependent, cell-based therapies more accessible in the future.

P257

Enhancing a gene-editing approach to express anti-HIV broadly-neutralizing antibodies in human B cells for HIV immunotherapy

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Antiretroviral therapy has considerably improved the management of HIV/AIDs but does not eradicate the viral reservoir, and a definitive cure for HIV infection has never been achieved. Immunotherapies represent novel treatment options that could prevent viral re-emergence. Broadly neutralizing antibodies (bnAbs) which have been identified in some patients, may be useful to develop novel immunotherapy or vaccination strategies. Recently, CRISPR/Cas9 gene editing has been used to genetically engineer the antibody repertoire of human B cells, providing a possibility to durably express high affinity HIV bnAbs in patients. We have established a non-viral gene editing protocol to modify the antigenic specificity of human peripheral blood B cells and to express HIV-specific Abs. Homology-directed repair templates coding for HIV bnAb H and L chain variable regions (in different configurations), was inserted in the human IgH gene to be spliced into IgH constant regions and to produce a membrane-bound or secreted Ig with the desired antigenic specificity. The single strand DNA template was produced by an optimized PCR

with increased yield and purity. Gene editing efficacy and specificity was validated in cell lines and in human primary B cells. Typically, in 3 days, 1-2% of primary human B cells can express membrane bnAbs 3BNC117 and VRC01 which target the HIV-1 env CD4 binding site. Human blood B cells edited to express the 3BNC117 bnAb show evidence of proliferation after antigenic stimulation with HIV viral-like particles, providing a rationale to combine B cell antigenic-specific preprogramming with vaccination to boost immune responses. Novel bnAbs targeting different sites on the HIV-1 envelope were identified and cloned by our team, providing novel paratopes for broad HIV neutralization and protection. We are also exploring new methods to increase gene editing efficiency. The DNA-PK inhibitor M3814 was found to significantly enhance 3BNC117 bnAb HDR template insertion in RAMOS B cells by 3.5 fold (p=0010, n= 3) with limited toxicity on the cells. Further experiments are ongoing in edited primary B cells in vitro and in vivo. Thus, gene editing HIV env-specific bnAbs in B cells may represent a useful gene therapy approach for HIV immunotherapy.

P258

Vectored long-term antibody delivery for COVID-19 prophylaxis

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Pandemics of viral infections are a major threat to global health. The COVID-19 pandemic brought leaps in scientific discoveries of novel treatments and vaccines. However, immunocompromised patients are at an increased risk of severe COVID-19 but have the least effect of vaccinations. Currently available treatments are costly and impractical for long-term prophylaxis in this population. Vectored immunoprophylaxis offers long-term prophylaxis after a single injection by delivering a monoclonal antibody through a viral vector. Adeno-associated virus (AAV) is being used as a gene delivery platform in treatment of several diverse diseases and an increasing number of AAV-based therapies are being marketed. AAV is an ideal vector due to low immunogenicity, particularly in immunocompromised patients, serotype-dependent transduction of specific tissues and high transgene expression. We developed an AAV8 vector encoding the anti-SARS-CoV-2 monoclonal antibody A23-58.1. AAV treatment of mice elicited sustained antibody expression 12 weeks after treatment and effectively protected the mice after SARS-CoV-2 exposure with AAV-treated mice having significantly lower viral load and replicative virus in the lungs. This strategy offers a practical prophylaxis of at-risk populations with diminished vaccine responses and can be tailored against several additional viral infections with high mortality and morbidity.

P259

Predicting antiviral immune responses using T cell receptor repertoire analysis and AI

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T cells are crucial components of our immune system, driving both effector and helper functions in immune responses against foreign antigens while acting as the immunological memory of our body. T cells also play an important role in driving the antigen-specificity of an immune response through recognition of specific antigen-derived epitopes using the T cell receptor (TCR). In recent years and driven by the fast-paced evolution in next-generation sequencing, the analysis of TCR repertoire data has gained interest as a new source of information to increase understanding of T cell response diversity and dynamics in health and disease. TCR repertoires can be easily sequenced from any blood (low volume) or tissue sample, hold great potential for patient stratification and could be used to predict and monitor a therapy response or disease progression in a patient. For many therapies, including certain types of vaccines, gene therapies or anti-cancer treatments, it remains challenging to predict whether an individual will be a responder or non-responder. This difference in response to a therapy or disease is largely due to the fact that each individual's immune system is unique.

We have developed a platform based on T cell receptor (TCR) repertoire analysis driven by machine learning and artificial intelligence to detect antigen-specific T cells in blood or tissue samples. In this study, we present data validating our approach for immune monitoring of hepatitis B-specific responses against a commercial vaccine in an in-house clinical trial. Thirty-four people who were never exposed to hepatitis B or vaccinated against it participated in the study. These individuals provided blood samples before vaccination, with 2 doses of the hepatitis B vaccine, and at 3 time points afterward. We used high-throughput sequencing of memory CD4 TCR repertoires and machine learning annotation to show that individuals with pre-existing vaccine-reactive memory CD4 T cells elicited earlier and higher antibody titers and mounted a more robust CD4 T cell response to hepatitis B epitopes. In addition, integration of TCR sequence patterns into a hepatitis B epitope-specific annotation model can predict which individuals will have an early and more vigorous vaccine-elicited immunity. Thus, the presence of pre-existing memory T cell clonotypes has a significant impact on immunity and can be used to predict immune responses to vaccination.

These findings help to better understand how people develop immunity to pathogens, may guide the development of better vaccines and allow to predict who will develop immunity after vaccination. Our data show the feasibility of using TCR analysis though AI to annotate, classify and track disease-specific T cells. The platform was developed at the University of Antwerp and is spun-out in the start-up ImmuneWatch. We are currently working on the application of predictive biomarkers based on TCR repertoire analysis in other areas beyond infectious disease vaccines.

P260

CRISPR-Cas therapy towards a cure for HIV/AIDS

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Many potent antiviral drugs are currently available to treat HIV infection. However, antiviral therapy must be continued lifelong because HIV rebounds from an established reservoir when treatment is interrupted. HIV DNA can be targeted by the CRISPR-Cas genome editing tool. Our goal is to define a robust and safe combinatorial CRISPR-Cas regimen for an "HIV cure for all" that can disable diverse HIV strains in various cellular backgrounds.

By using a combinatorial CRISPR-Cas attack against conserved HIV sequences, we were able to cure HIV-infected T cells in vitro. Although remarkable, the large size of CRISPR-spCas9 transgene cassettes impedes their implementation in gene therapy applications with vectors that have a limited packaging capacity, including lentiviral vectors (LVs). There is a serious need for more simple/smaller CRISPR-Cas vector designs. We propose to minimize the size of the lentiviral vector by: a) adopting the small H1 Pol-III promoter that we recently found to exhibit both Pol-III and Pol-II promoter activity for the production of both the gRNA and Cas9-encoding mRNA and b) incorporating a smaller Cas nuclease. These measures will reduce the vector size and likely increase the vector titer.

We have compared the different CRISPR-Cas systems for their efficiency in terms of antiviral activity and viral titer. Virus inhibition was tested in HIV replication studies which allow us to test for viral escape (in long-term cultures) and a potential CURE of the infected cells. The viral gRNA-targets were sequenced to elucidate the mechanism of viral escape or the means of provirus inactivation in the case of a CURE. Superior antiviral activity is reported for saCas9 compared to cjCas9, which can achieve full HIV inactivation in cell culture with only a single gRNA. We also disclose that DNA cleavage by the saCas9 and cjCas9 endonucleases and subsequent DNA repair cause mutations with a sequence profile similar to spCas9. We demonstrated that a reduction of the vector size (smaller Cas9 nuclease and dual-polymerase active H1-promoter) increases the vector titer. This greatly facilitates the use of viral vectors with a limited packaging capacity.

However, delivering the CRISPR-Cas antivirals to the HIV reservoir cells is the major challenge. Untargeted conventional vectors may raise safety concerns because numerous particles will be absorbed by cells that are not relevant to the therapy, particularly when the therapy-relevant cells comprise a relatively small population, as is the case with latently HIV-infected cells. The molecule CD32a was proposed as one marker of the HIV reservoir and cells with a very high enrichment in viral DNA. Our focus is to evaluate the impact of a CRISPR-Cas attack on those cells. The lack until now of an antibody to accurately distinguish between the CD32 isoforms has precluded the development and testing of a CD32a-molecularly targeted approach. We have developed LVs with a specific tropism for CD32a+ CD4T cells by engineered viral vectors displaying Designed Ankyrin Repeat Protein (DARPins) for the target receptor and cell type of choice. These results are important on the path towards formulating a cure strategy.

P261

Adeno-associated viral vectored delivery of monoclonal antibody genes against blood-stage malaria

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Malaria remains one of the most devastating infectious diseases, resulting in the death of just under half a million individuals every year. Despite extensive efforts, the development of a highly efficacious and durable vaccine has proved exceedingly difficult, and new and innovative intervention strategies are likely to be needed. Following parasite release from the liver, blood-stage vaccines aim to reduce mortality, clinical disease, and transmission, whilst potentially also allowing for natural boosting of vaccine-induced responses and the acquisition of natural immunity. However, a significant challenge in the development of a blood-stage malaria vaccine is

the need to induce, and maintain, the very high levels of antibodies necessary to neutralise the parasite's very rapid invasion of red blood cells.

An alternative approach to obtain the required humoral immunity against blood-stage malaria is to use potent monoclonal antibodies (mAbs) as prophylactics, which would bypass the need for a vaccine to aid in current malaria elimination programmes. Vectored immuno-prophylaxis (VIP) uses viral vectors such as adeno-associated virus (AAV) to deliver mAb-expressing genes, which are expressed *in situ* following immunisation and released into the plasma. In the last few years we have isolated panels of human mAbs targeting *Plasmodium* blood-stage antigens from vaccinated volunteers, including potently neutralising and synergistic antibodies. In this study, we deliver fully human, potently neutralising mAbs by VIP in mice, and we achieve durable and high-level serum mAb expression that is strongly inhibitory of parasite growth. This approach, combined with anti-malarial drugs and other control interventions, could provide an effective strategy towards the ambitious objective of malaria eradication.

P262

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 talk 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

P263

Cell-based proteolytic sensing systems: platforms for vaccine and antiviral development

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One of the greatest public health challenges is the incidence of infectious diseases. Although the improvement of sanitary conditions, the establishment of quarantines, and rigorous vector control have been used to suppress viral diseases, vaccination and antiviral therapies are the main successful measures for the prevention and treatment of viral diseases, respectively. Their development relies on quantification and detection of infectious viruses, which are performed through inaccurate and/or very time-consuming methods. This hinders research in applied virology and, consequently, the development of vaccination and antiviral therapies.

A common feature of most viruses is the presence of one or more proteases, enzymes that hydrolyse peptide-amide bonds. These enzymes are essential for their replication and survival, holding tremendous potential as biomarkers of viral disease. Therefore, proteolytic detection systems can improve basic virology research methods and enable the development of antiviral therapies. Herein, a novel cell-based system, genetically encoded, for detecting viral proteolytic activity is described.

The sensing system consists of (1) a structurally distorted Cre recombinase, through cyclization by *Nostoc punctiforme* DnaE intein splicing **(inactive state),** harboring a protease cleavable sequence as sensor, and (2) a reporter cassette encoding an inverted GFP inserted into flip-excision system. Therefore, in the protease presence, distortion is relieved **(active state)**, resulting in stable fluorescence emission. Herein we show the successfully development of this sensor to detect *Tobacco etch virus* (TEV) and *Human Rhinovirus* (HRV) in H1-HeLa cells.

H1-HeLa cells genetically encoding the reporter module (H1-HeLa$^{Dio\text{-}meGFP}$) were developed and tested by co-transfection with two plasmids expressing the sensor module and HRV or TEV protease to evaluate sensor performance. Positive and negative controls were also performed to determine the maximum sensor signal and background, respectively. Through flow cytometry and fluorescence microscopy analysis, 70% of GFP$^+$ cells for the TEV biosensor, and 60% of GFP$^+$ for the HRV sensing system were obtained 24 hours after transfection. These results were similar to the positive controls. Values of less than 2.5% of GFP$^+$ cells were achieved for both sensor systems for the background controls. Therefore, these preliminary results show robust event detection and low of background activity, resulting in good sensor performance. Currently, H1-HeLa$^{Dio\text{-}meGFP}$, encoding also the sensor module for both TEV and HRV, are being developed and will be challenged through viral infection.

Due to the sensing system design, this system can be easily adapted to other emerging viruses, such as Severe Acute Respiratory Syndrome Coronavirus 2, West Nile virus, Chikungunya virus, or Zika virus. Moreover, this sensing system cannot only be used as a reliable quantification method, but also as a platform for the development of antiviral therapies. In addition, adapting the reporter module to express an effector protein enables the research of potential antiviral gene therapies. Therefore, this biosensor has the potential to revolutionize the development of vaccines and antiviral therapies.

P264

Induction of immunity against HBV following a single dose injection of HBV surface antigen-expressing adenoviral vector in mice

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Hepatitis B is not curable and results in 887 000 deaths worldwide every year. The protein subunit vaccines currently used for prevention of hepatitis B virus (HBV) infection are limited by the requirement of 3 doses to achieve optimal protection. In addition, they have poor efficacy in individuals who are older than 40 years or have certain medical conditions such as HIV infection or chronic renal insufficiency. Adenoviral vector (AdV)-based vaccines have recently shown promise in preventing viral infections such as that caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Unlike protein subunit vaccines, AdV-based vaccines induce a robust cellular and humoral immune response. In this study we produced AdVs expressing either a secreted small or non-secreted large HBV surface antigen (SHBs or LHBs). Infection of cultured cells with surface antigen expressing AdVs resulted in a significant dose-dependent expression of SHBs or LHBs. SHBs levels were significantly higher in both cell lysates and supernatant. As expected, LHBs levels were significantly higher in the lysates and not in the supernatant. Current studies are assessing the ability of these AdVs to induce HBV-specific and neutralizing immune responses in mice. The fact that 1.5 million new HBV infections still occur in the world annually emphasizes the urgency to address the shortcomings of current vaccines. AdV based vaccines may simplify implementation of vaccination programs and improve compliance by enabling single dose vaccination. They may provide an alternative or the only option for the 5 % of the world's population not protected by the existing anti-HBV vaccines.

P265

Directed evolution of Adenoviruses to improve vectored vaccines properties

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Replication incompetent Adeno (Ad)-based vectors are able to induce potent adaptive immune responses in humans, being of interest for infectious diseases and cancer therapy or prevention. Several Ad-based vaccines have already been approved, also during the Covid-19 pandemic.

Immunological potency of Ad-vectors from different serotypes can be influenced by the extent of pre-existing host immunity, different cell tropism and intracellular trafficking, also impacting the extent of innate immunity induction.

We have set up a procedure allowing the generation of libraries of Ad-vectors genomic variants with high diversity and successfully applied this technology to the hypervariable regions (HVR) of the hexon, the main Ad neutralization determinants also involved in virus intracellular trafficking.

HVRs were mutagenized through error prone PCR and transferred into a vector genome BAC construct with an optimized recombineering protocol; an improved rescue procedure was applied for the recovery of all available variants.

Seven Adenoviral genome libraries were generated with mutations in single or all HVRs, leading overall to more than 10^6 different genomic variants. Such libraries were successfully rescued and then subjected to different selective pressures. Characterization of variants isolated in different conditions is ongoing and will focus on growth properties, recognition by anti-Ad antibodies present in humans and immunological potency.

We have developed a tool for generation of Adenoviral genome variant libraries ideally allowing to target any genomic region of interest, providing unprecedented versatility. This open the way to identification of novel vector variants with improved properties for both prophylactic and therapeutic vaccine applications.

P266

Enhancement of the anti-tumor effectiveness of whole-cell cancer vaccines through different injection methods

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It is not uncommon for cancer to reoccur despite successful treatment of the primary tumor. Recurrent or metastatic cancers often exhibit lower responsiveness to existing therapies and have a high mortality rate. Utilizing patient-derived primary tumor tissue, whole-cell cancer vaccines have shown promise in preventing recurrence. One significant advantage of this approach is the abundance of patient-specific tumor antigens it provides. However, the overall efficacy of whole-cell vaccines remains limited and insufficient. To address this, we investigated the utilization of different injection methods to enhance the effectiveness of whole-cell cancer vaccines. By comparing the efficacy of needle-syringe injection and needle-free injection (pyro-drive jet injector), we evaluated the varying effectiveness of whole-cell cancer vaccines using murine MC38 colon adenocarcinoma or 4T1 mammary tumor cell lines. We assessed tumor challenge and the inhibition of cancer metastasis using C57BL/6 and BALB/c mouse models. Additionally, we analyzed the in vitro activation of antigen-presenting cells (APCs) and innate immune cells through flow cytometry, cytokine array, and quantitative real-time PCR, employing the expelled whole-cell vaccine following each injection method. Our findings demonstrated that needle-free injection of the whole-cell vaccine induced a higher tumor suppression effect and stronger tumor-specific immunity compared to traditional needle-syringe injection in both MC38 and 4T1 tumor models. In vitro experiments revealed that the whole-cell cancer vaccine preparation discharged through the needle-free injection device triggered a more robust immune stimulation in immune cells compared to the syringe method. In contrast to needle injection, the needle-free injection of the whole-cell cancer vaccine led to the generation of more pulverized cancer cell fragments. This, in turn, enhanced the phagocytosis of APCs and promoted adaptive immune activation, ultimately improving the vaccine's anti-tumor effect. Consequently, the adoption of needle-free injection or pre-pulverized formulations should be considered to enhance the anti-tumor effects of whole-cell cancer vaccines.

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CRISPR Cas13 as an antiviral tool against respiratory RNA viruses

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In 2019, a new emerging Coronaviruses (SARS-CoV-2) transmitted zoonotically from bats to humans, leading to the recent pandemic. Although immunity in large parts of the population induced by infections and vaccinations decreased the disease burden of COVID-19, investigations towards new therapeutics, drugs and vaccines targeting not only SARS-CoV-2 but also against various Coronaviruses, both with zoonotic potential such as MERS and yet to be transmitted sarbecoviruses in bats are ongoing. In this project, we harness the CRISPR Cas13 system recently discovered as an RNA-dependent endonuclease slicing phage-derived RNAs. Cas13 can be trained to target the SARS-CoV-2 RNA genome specifically directly after cell entry, inhibiting the initial round of translation and thus RNA replication cycle. Furthermore, alignment of three common known Coronaviruses including HCoV-229E, SARS-CoV and SARS-CoV-2 enable us to design gRNAs against conserved sequences, generating one tool for the restriction of several Coronaviruses in parallel. So far, we could confirm Cas13 restriction for a cellular mRNA by transfecting RfxCas13d and gRNAs against CXCR4 into a 293T cell line overexpressing this receptor. Here, we observed up to 60 % downregulation of the receptor on the cell surface. Furthermore, we established an additional proof of concept model using 293T cells, stably expressing the HCoV-229E receptor CD13, linked to a Firefly luciferase (Fluc) as a reporter for cell viability. By designing gRNAs against Renilla Luciferase (Rluc) encoded by the HCoV-229E virus itself, we tested RfxCas13d for its restriction efficacy in a viral context. We tested different delivery methods of Cas13 and gRNAs: plasmid transfection, lentiviral transduction, Cas13 mRNA and synthetic gRNA delivery, respectively. In the latest case, restriction of Rluc signal reached the highest efficiency in the context of HCoV-229E-Rluc reporter virus with up to 75 % reduction. Efficient restriction was confirmed by RT-qPCR on two subgenomic viral RNAs of the HCoV-229E-Rluc reporter virus. This strategy enabled us to differentiate between restriction of the incoming coronaviral genome and subgenomic RNA replication, respectively. In the future, we plan to target conserved regions and useSARS-CoV-2 in a BSL-3 set up as the challenging virus. The most successful Cas13/gRNA payload will be delivered *in vivo* using viral or non-viral vector to validate their antiviral activity upon SARS-CoV-2 infection. Adeno-Associated Virus serotype 6 (AAV6) vectors including capsid mutations enhancing targeting to the lung were already evaluated for functionality and safety using luciferase expression in BALB/c wt mice via intratracheal and intranasal instillation. Both vectors showed robust firefly luciferase expression exclusively in the thorax (lung) region 1 week and 5 weeks post administration.

P268

Synthetic eye organoid-based disease modelling for gene therapy of Bietti's crystalline dystrophy

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Bietti's crystalline dystrophy (BCD), is a progressive chorioretinal degenerative disease with *CYP4V2* mutation which eventually result in vision loss and blindness in patients. At present, there is no cure for BCD due to a lack of suitablehuman-derived BCD disease models for meaningful exploration of the disease mechanisms and potential therapeutic strategies. With the recent advances in genome editing technology, gene therapy has been regarded as a promising means to cure BCDs. Up to now, animal models have been used to study disease mechanisms and perform therapeutic testing for BCD. However, interspecies anatomical differences, high costs and ethical issues make these models suboptimal for this purpose. Recent advances in induced pluripotent stem cell (iPSC) technology have provided an excellent opportunity for us to derive disease-specific iPSCs from patients and create disease-relevant 2D and 3D derivatives for BCD modeling *in vitro*. In this study, we utilized BCD-patient-derived iPSCs carrying a familial *CYP4V2* mutations/deletion to model BCD in vitro. Our results demonstrated that the BCD-iPSC derived retinal pigmental epitheliums (RPEs), photoreceptor cells recapitulated BCD-associated phenotypic events. In addition to the 2D cell cultures, we created a 3D retinal organoid system for BCD disease modeling. Characterization of BCD-associated disease phenotypes showed that lipid droplet accumulation occurs in BCD-hiPSC-derived retinal organoid cells, and both RPE and photoreceptor cells within the organoids were prone to cell death. Furthermore, we have used prime editing to convert the *CYP4V2* mutations and deletion followed by various BCD-iPSC-based cellular and functional assays. Our results shown primate editing convert the *CYP4V2* mutations and deletion, reduced the RPE degeneration and restore the function of the BCD-iPSC-derived eye cells and organoids. These findings demonstrate the potential of prime editing treatment for BCD.

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Primitive Human iPSC-Derived Macrophages with Enhanced Functionality to Eradicate Mycobacterial Infections

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Mycobacterium tuberculosis (*Mtb*), as the main pathogen for the cause of lung tuberculosis (TB), still continues to be a global threat to the public health. With more than 11 million new infections and 1.6 million deaths per year, TB is the 2nd leading cause of death within the class of infectious diseases. Although the current standard treatment using a combination of antibiotics is effective, the constant mis- and overuse is the main driver for antibiotic-resistant strains, limiting the

number of compounds available for successful future treatment. Moreover, *Mtb* developed evasion strategies, which enable the pathogen to escape the intra-cellular defense mechanisms by skewing the function of alveolar macrophages. Modern immunotherapeutic approaches are directed to interfere with the aforementioned evasion strategies, aiming at the activation of macrophages by different approaches. Besides these promising strategies, another alternative would be the generation of enhanced macrophages, which could be used directly as an off-the-shelf cell-based immunotherapy.

As a first step towards this direction, we investigated the immunological response and functionality of primitive macrophages, which are derived from human induced pluripotent stem cells (iMacs). Generation of iMacs has been performed from two different healthy individuals and the response post-infection with *Mtb*, heat-killed *Mtb* (HKMT) or the attenuated TB strain Bacillus Calmette-Guérin (BCG) has been analyzed. Of note, classical monocyte-derived macrophages (MDMs) have been used as a control.

Herein, we show that both types of macrophages start phagocytosis of HKMT shortly after contact with iMacs demonstrating a stronger migration and cluster formation towards HKMT and an overall faster and enhanced phagocytosis rate compared to MDMs. In addition, iMacs showed a stronger downregulation of activation markers CD16 and TLR2, which are known to play a crucial role in the defense of mycobacterial infections. Furthermore, iMacs demonstrated a higher increase of pro-inflammatory cytokine response upon infection with HKMT, BCG or *Mtb* compared to MDMs, including a subsequent downregulation of the pro-inflammatory state, thus, being able to return much faster to the baseline cell activation, indicating an overall stronger and faster immune response. Of note, both, iMacs and MDMs, demonstrated a downregulation of markers for the maturation of phagosomes upon infection and thus, evading strategies to prevent proper phagocytosis of BCG. However, iMacs showed a clear localization of BCG and V-ATPases, indicating a strong phagosome acidification post-infection. In fact, both types of macrophages were still able to eradicate *Mtb*, while iMacs showed a stronger killing capacity.

We here reveal iMacs of primitive, pluripotent origin with enhanced functionality to successfully eradicate mycobacteria, which may be considered for novel therapeutic applications.

P270

An NGS-based and marker-free CRISPR pipeline speeds up the creation of knock-in zebrafish and hiPSC disease models

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The rearing and selection process of correctly CRISPRed zebrafish and human induced pluripotent stem cell (hiPSC) clones remains time-consuming. Often fluorescence or antibiotic markers are applied but in general the average *knock-in* (KI) efficiency in these models is low (<5%) when using the conventional CRISPR/Cas9 system. Hence, many fish need to be reared at random while only a fraction potentially passes the edit on to their offspring. Analogously, many hiPSC clones need to be individually screened to increase the chance of finding the desired KI clone. To functionally assess genetic variants identified in Brugada syndrome (BrS) patients we aimed to develop a

marker-free, Next Generation Sequencing (NGS)-based CRISPR workflow to facilitate fast and efficient CRISPR editing, potentially applicable in a diagnostic setting. To create a BrS KI zebrafish and hiPSC line with the same missense variant, different CRISPR components were tested to obtain the combination with the best KI efficiency. Variations of sgRNA and Cas9 protein, forming a ribonucleoprotein (RNP) and single-stranded oligodeoxynucleotides (ssODNs) were assessed. The components were injected in fertilized zebrafish eggs at the one cell stage. At three days post fertilisation, DNA of each individual larva was obtained while keeping them alive by using the zebrafish embryo genotyper. Upon targeted NGS (MiSeq) evaluation, only the embryos with ≥2% perfect KI reads were reared. This reduced the total animal number significantly and the time needed to find the mosaic fish with the desired germline mutation, as only around 20-25% of the screened larvae are eventually reared. In the hiPSCs, the components were introduced with nucleofection followed by a low density seeding. Only the wells that contained the cell population with the highest KI%, confirmed by targeted NGS (MiSeq), were kept in culture for further validation. The best CRISPR components combination had an average KI% of 2.99 (± 5.09, N=122) in the zebrafish. One in six mosaic fish passed on the mutation and both hetero and homozygous lines were bred. For the hiPSC, the best CRISPR components combination achieved a KI% of 64.2 (± 27.1, N=21 wells). Interestingly, the most efficient ssODN conformation was also the most efficient in the zebrafish. Hence, homo- and heterozygous KI BrS hiPSC clones were successfully obtained. Moreover, the efficiency of this NGS-based CRISPR workflow was validated by introducing a missense mutation in human embryonic stem cells (hESCs) that is associated with a range of neurodevelopmental disorders. For the most optimal CRISPR system an average KI efficiency of 50.2% (± 24.8, N=46 wells) was achieved. Lastly, in both hiPSC and hESCs, a low density seeding of 10 cells per well will already give precise homo or heterozygous KI cell populations in 24% (N=79) of the screened wells after NGS analysis compared to only 6% when 50 cells per well were seeded (N=124). We showed that with our customised NGS workflow, we can successfully create KI lines in a marker-free way, irrespective of the study model. Due to parallel NGS screening, the overall time needed to obtain the desired KI line is reduced.

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Engineering human iPS cells and their myogenic derivatives for advanced in vitro modelling of Duchenne muscular dystrophy

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Muscular dystrophies are a heterogenous group of genetic disorders characterised by progressive skeletal muscle degeneration. Notably, several muscular dystrophies also have cardiac muscle involvement, such as Duchenne muscular dystrophy (DMD), the most common paediatric muscular dystrophy. On the other hand, this cardiac phenotype is poorly recapitulated in most animal models, which primarily focus on skeletal muscle abnormalities. Therefore, we aim to: 1) utilise DMD patient-specific human induced pluripotent stem cells (hiPSCs) to derive cardiac and skeletal muscle cells to examine disease pathogenesis; 2) recapitulate the intricate multicellular tissue structure of striated muscle through 3D engineered tissue systems to enhance *in vitro* maturation; 3) establish DMD *in vitro* phenotypic readouts in hiPSC-derived models to test

therapeutic strategies. To achieve these objectives, DMD and healthy control hiPSCs were differentiated into cardiac and skeletal myogenic cells using a transgene-free protocol and then combined with biomaterials into 3D engineered muscle tissues (engineered heart or skeletal muscle tissues, EHTs and ESMTs). Molecular analyses were performed to assess tissue differentiation and maturation, whilst excitation-contraction coupling was studied by calcium imaging. Gene expression analysis indicated enhanced tissue maturation in EHTs vs conventional cardiomyogenic monolayer cultures. The electrophysiological analysis demonstrated contraction abnormalities in DMD models resembling *in vivo* disease phenotype. A reduction of cardiomyogenic cells with a concomitant increase of fibroblast-like cells was detected in DMD EHTs vs. healthy controls. Moreover, we have combined the aforementioned tools with new isogenic DMD and healthy hiPSC lines containing a reporter cassette to allow real-time tracking of dystrophin expression dynamics and localisation in both striated muscles in a humanised 3D platform. Finally, we will discuss the application of these advanced *in vitro* disease modelling platforms for development and validation of DMD-targeted genetic therapies.

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Development of iPSC-based models to test microglia-directed gene therapy for neurodegeneration

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Deficiency of progranulin (PGRN), caused by mutations in the granulin (*GRN*) gene, is responsible for two fatal neurodegenerative diseases: neuronal ceroid lipofuscinosis-11 (CLN11) in children and young adults, and frontotemporal dementia (FTD) with typical onset before 65 years of age. Currently, no treatment is available to prevent or slow neurodegeneration in these patients. Since microglia are critically involved in the pathological process, we developed an *ex vivo* hematopoietic stem and progenitor cell (HSPC) gene therapy that targets this cell type. We designed a lentiviral vector (LVV) that drives phagocyte-specific PGRN expression to reconstitute PGRN secretion in macrophages and microglia. In an autologous setting, the patient HSPCs are transduced *ex vivo* with the gene therapy LVV and reinfused upon myeloablation. Graft-derived myeloid cells engraft the brain and generate new microglia-like progeny able to secrete higher levels of PGRN.

We tested a series of LVVs carrying different candidate promoters in myeloid cell lines such as THP-1, and in macrophages differentiated from healthy donor HSPCs. The LVV conferring the highest expression in myeloid cells was next tested in an induced pluripotent stem cell (iPSC) microglia model derived from a *GRN*-FTD patient. To confirm the identity of these cells, we

verified the expression of typical microglia markers such as IBA1, TMEM119 and SALL1. Upon transduction of the *GRN*-FTD iPSCs and microglia differentiation, both PGRN expression and secretion increased to physiological levels.

To mimic the integration of PGRN-enhanced microglia in the patient's brain, other brain cell types need to be included in this model. Therefore, we are developing an *in vitro* co-culture system consisting of neurons, astrocytes and microglia, all differentiated from the same iPSC line carrying pathogenic *GRN* mutations. In a pilot experiment, we defined the culture medium able to support the differentiation and survival of all three cell types. We observed that iPSC-derived microglia integrate in the network of neurons and astrocytes, and develop the characteristic ramifications found *in vivo*. In this setting, we will next investigate the effect of PGRN reconstitution, secreted by gene-modified microglia, on the other brain cell types, as well as the correction of typical disease phenotypes such as lysosomal dysfunction. The development of this iPSC-based brain co-culture model represents a fundamental innovation in the study of PGRN-deficiency and the associated neurodegeneration. It will allow not only to test candidate LVVs for microglia gene therapy in a human context, but also to investigate the human-specific molecular mechanisms of *GRN*-related neurodegeneration.

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Generation of an iPSC-derived hepatic organoid for liver cell therapy

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The only curative treatment for chronic liver diseases and for several inborn metabolic diseases is liver transplantation but it is limited by the shortage of organs. Currently, primary cryopreserved hepatocytes are the gold standard for liver cell therapy, but they have several limits, such as low cell renewal, loss of their phenotypic characteristics and functions once isolated, and poor engraftment in the host liver. In order to overcome this issue, we are developing transplantable liver organoids mimicking the complex structure and function of the liver. In particular, our aim is to develop a hetero-cellular spheroid system composed of hepatocyte-like cells, hepatic stellate-like cells and endothelial-like cells derived from human pluripotent stem cells (iPSC). So far, we obtained a homogeneous population of epithelial cells expressing the endoderm marker SOX17 and the early hepatic marker HNF4α. The differentiation of these hepatic endoderm cells was continued *in vitro* in a 3D structure in the presence of iPSC derived endothelial like cells expressing vWF and CD144 and stellate-like cells expressing PDGFR-β and vimentin. The paracrine signals derived from neighbouring endothelial and hepatic stellate cells are likely to increase hepatic maturation and functions. One of the major drawbacks of using these potential ATMP (Advanced Therapy Medicinal Products) for liver cell therapy is the need to generate patient specific cells in order to avoid immune rejection which leads to their rejection. However, the autologous cell therapy is a time consuming and costly process and it is very challenging for inborn liver diseases carrying the disease-causing mutations that must be corrected in advance. In order to avoid any immune-rejection, we have generated a heterologous hypoimmunogenic iPSC (hypo-iPSC) cell line that will be available to all patients by ablating HLAI and HLAII expression. In this study, we employed the CRISPR/Cas9 system to delete the *B2M* and *CIITA* genes, essential for the correct surface expression of HLA-I and HLA-II proteins. The resulting hypoimmunogenic iPSC line has a normal karyotype, expresses the pluripotency stem cell markers, and is capable of differentiating into the three embryonic germ layers. Furthermore, we showed that it specifically

retains the ability to differentiate towards endothelial-like cells, hepatocyte-like cells and stellate-like cells.

Our results pave the way for the generation of an hypoimmunogenic liver tissue with the intrinsic ability to survive long-term in a fully allogeneic recipient without immunosuppression, representing a promising cost-effective and off-the shelf cell therapy for liver diseases.

P275

Human stem-cell based models to study innate immunity and neuroinflammation in the central nervous system

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The development of viral-based gene therapies for central nervous system (CNS) disorders has been hindered by the host immune response. Upon infection, glial cells trigger an innate immune response which will mediate the signaling for immune cell infiltration, leading to neuroinflammation and, consequently, neuronal death. The mechanisms that govern the crosstalk and sustain this neuroinflammatory process in human glial cells remain largely unknown. In recent years, human induced pluripotent stem cell (hiPSC)-derived three-dimensional (3D) models leveraged the study of complex brain/CNS biological processes. These 3D structures, in which different brain/CNS cell lineages are represented, can contribute to decoding the molecular mechanisms that trigger and sustain glial activation and drive microenvironment remodeling in the human CNS. However, there are still significant caveats with current 3D hiPSC-derived models, such as high heterogeneity, usage of animal-derived components, differentiated cells with an immature or inflammatory phenotype, and limited functionality. Here we propose a human innate immunocompetent CNS model that recapitulates neuroinflammatory hallmarks and is a suitable platform for preclinical development. hiPSC-derived neural progenitors were cultured in perfusion stirred-tank bioreactors and differentiated into 3D neurospheroids composed of neurons, astrocytes, and oligodendrocytes. A co-culture between hiPSC-derived microglia (iMGL) and neurospheroids was established to mimic the neuro-immune axis. iMGL infiltrated the neurospheroids, retaining expression of IBA1, TMEM119, and P2RY12, amongst other microglia markers, and their morphology ranged from ramified to amoeboid. Neurospheroids, alone or co-cultured with iMGL, were challenged with LPS and IFNγ to induce neuroinflammation. These stimuli lead to the secretion of chemoattractants in the neurospheroids, mainly ligands of CCR3 and CCR4, while in co-cultures, we observed secretion of a broader spectrum of proinflammatory mediators of TNF and NF-kB signaling, concomitant with an upregulation of proinflammatory genes (e.g., IL-8, CCL2, and TNF). Adeno-associated virus (AAV) serotype 9, a candidate for gene therapy able to cross the blood-brain barrier, was used to assess viral vector immunogenicity. Neurospheroids were transduced with rAAV9-eGFP at 5 x 105 VG/cell, and the transgene expression and inflammatory response were evaluated. rAAV9-eGFP transduced both neurospheroids without and with iMGL showing a higher tropism towards neurons. This challenge led to the upregulation of IL-6 and CXCL8 in the first and GFAP in the latter. Hence, we propose the human neurospheroid model as a useful preclinical model to accelerate the development of advanced therapies.

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CRISPR/Cas9 disruption of DNA synthesis as a tool to control cell proliferation in vitro and in vivo

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Robust proliferation is a key feature of most cells used in scientific research and biotechnology. Some of them, like induced pluripotent stem cells (iPSC), additionally hold a lot of promise for regenerative medicine and cell/gene therapies, as they can differentiate into functional, potentially therapeutic tissues. However, most living cells bring about a fundamental biological risk of unlimited or unwanted growth that limit their use in clinical settings. Here, we describe a novel metabolic safety system to control cell proliferation omitting the use of transgenic elements. Using CRISPR/Cas9, we inactivated the enzyme in charge of the de novo thymidylate synthesis (TYMS) in several cell lines. This resulted in cells that proliferate when supplemented with exogenous dTMP but fail to grow in its absence. As thymidine is needed for DNA, but not RNA synthesis, we show that this approach targets only proliferative cells, without affecting their postmitotic function. Thus, the method allows robust cell culture and manufacture while diminishing the risk of uncontrolled growth of transplanted cells.
Under dTMP supplementation, TYMS-/- iPSCs maintain their pluripotency and differentiate normally into potentially therapeutic cell types, e.g. pancreatic beta cells in vitro. In vivo, they produce teratomas, the size of which reflects the dTMP supplementation. Most importantly, after terminal differentiation, TYMS -/- hiPSC derived beta cells no longer require dTMP to remain functional, as seen by prolonged in vivo production of human insulin and subsequent decrease in blood glucose levels in mice.

P277

Epigenome-editing strategies to enhance oligodendroglial differentiation of human induced pluripotent stem cells

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The generation of human induced pluripotent stem cell (hiPSC)-derived oligodendroglial progenitors (OPC) and oligodendrocytes (OL) at yield, purity, and homogeneity required for translational applications in demyelinating disease is still a challenge. Current protocols to generate hiPSC-derived OPC/OL are time-consuming, lead to heterogeneous cell populations, and could lead to gene misregulation and/or genotoxicity if based on stable overexpression of OPC/OL-related Transcription Factors (TFs).

Here, we exploited novel epigenome-editing technologies to timely and physiologically modulate the expression of OPC/OL-related genes in human iPSC-derived neural stem cells (hiPSC-NSCs) through Artificial Transcriptional Activators (ATAs) based on dCas9 fused to transcriptional

activator domains. After the selection of target regulatory regions, we confirmed the capability of ATAs to robustly and transiently up-regulate key OPC/OL genes in hiPSC-NSCs, which were then differentiated using an established differentiation protocol resulting in OPC/OL-enriched cultures. Gene expression, cytofluorimetric, and immunofluorescence analysis showed enhanced expression of OPC/OL markers and increased numbers of OPC/OL with enhanced branched morphology in epigenome-edited cultures as compared to untreated (UT) controls, suggestive of enhanced differentiation/maturation. Furthermore, we validated the tolerability of the epigenome-editing approach in hiPSC-NSCs derived from patients with genetic demyelinating diseases and corrected by LV-mediated gene therapy treatment. Upon intracerebral transplantation in immunodeficient neonatal mice, epigenome-edited OPC showed robust and long-term engraftment, widespread migration, and acquisition of a mature/myelinating OL phenotype, with no sign of aberrant proliferation/differentiation. When transplanted in murine models of demyelinating leukodystrophy, epigenome-edited OPCs were capable to attenuate the pathological phenotypes. The functional advantage of epigenome-edited OPC over UT counterparts is under evaluation.

The results of this study suggest that transient activation of the endogenous OPC/OL regulatory network in hiPSC-NSCs favors their oligodendroglial commitment/maturation and is well tolerated in combination with gene therapy and cell transplantation approaches in murine disease models. Overall, we gave proof-of-principle of feasibility, safety, and therapeutic potential of state-of-the-art epigenetic modulation technologies in hiPSC-NSCs to obtain functionally competent OPC populations that can be harnessed for basic and pre-clinical research.

P278

Modelling X-linked centronuclear myopathy using advanced human skeletal muscle models

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Centronuclear myopathies (CNM) are incurable congenital muscle disorders characterised by skeletal muscle weakness and atrophy. Histologically, CNM muscles have small myofibres and organelles (including nuclei) abnormally positioned in the centre of myofibers. The most severe CNM form is the X-linked centronuclear myopathy (XLCNM, also known as myotubular myopathy) caused by mutations in *MTM1* coding for Myotubularin, a lipid phosphatase involved in membrane remodeling and cytoskeleton organization. Myotubularin pathogenic role in XLCNM is still not fully elucidated. Current human *in vitro* models are based upon invasive muscle biopsies of infants, fail to recapitulate the complexity of human skeletal muscle and are not amenable for long-term culture. Moreover, other mammalian (*in vivo*) models have generally low throughput, are associated with ethical and financial issues and have sub-optimally predicted safety concerns which halted recent gene therapy clinical trials. Therefore, here we generated novel advanced human *MTM1*-mutant models by developing a *quasi-vivo* platform based upon bioengineering 3D muscles to investigate tissue architecture and function during XLCNM muscle development, degeneration and regeneration. XLCNM engineered muscles derived from immortalised myoblasts showed aligned myofibers and efficient skeletal myogenic differentiation and maturation. However, *MTM1*-mutant myofibres had reduced diameter compared to matched controls, recapitulating the fibre hypotrophy observed in patients. Functional assays were then performed to check if fibre hypotrophy was associated with defective excitation-contraction coupling.

Analysis of calcium dynamics upon electrical stimulation indicated no significant difference in calcium release in XLCNM engineered muscles compared to healthy controls. However, abnormal protein levels of ryanodine receptors were observed, also mirroring what observed in muscle biopsies. To assess for possible early developmental myogenesis defects, we generated an XLCNM iPSC line and differentiated it into myoblasts and myotubes using a transgene-free protocol: no differences in skeletal myogenic potential was observed between control and mutant iPSCs, indicating that mutations in *MTM1* do not impair human developmental myogenesis. We are now harnessing this new iPSC line alongside the validated immortalised myoblasts to expand the portfolio of disease phenotypic readouts (e.g. metabolic dysfunction) and to develop multi-cellular, -tissue and -organ models capable to assess toxicity and efficacy of emerging genetic therapies for XLCNM.

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A zebrafish model of macular cornea dystrophy provides the first preclinical test model

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Macular cornea dystrophy (MCD) is a rare disease that leads to the formation of aggregates in the corneal stroma which progressively blocks light. The disease is usually symptomatic at the age of 12 and patients suffer from complete loss of vision in both eyes by the age of 30-40. Being an autosomal recessive rare disease, the prevalence is higher in consanguineous populations including Iceland, South India, and Turkey. The only available treatment is cornea transplantation. Not only cornea donors are limited but also the disease relapses within 5 years of transplantation due to the invasion of the healthy cornea by host keratocytes. The MCD is linked to loss of function mutations in the carbohydrate sulfotransferase 6 (CHST6) gene, which encodes for an *N*-acetylglucosamine-6-*O*-sulfotransferase enzyme. The CHST6 is responsible for the sulfation of keratan sulfate proteoglycans (KSPGs). Unsulfated KSPGs aggregate in the stroma of the cornea. Up to date no in vivo model of the MCD was reported. Both the structure of the cornea and the chst6 gene are well conserved in zebrafish. Here, the first chst6 mutant model organism was generated by targeting the zebrafish chst6 gene via CRISPR/Cas9. Several mutant alleles were identified, and loss of function was confirmed via ELISA. Larval and adult eyes were stained with corneal keratan sulfate (cKS) specific antibody which showed complete loss of cKS in the corneal stroma, whereas epithelial markers were unchanged. Macroscopic examination showed a gradual increase of opaque corneal aggregates, and histological analysis showed alcian blue positive aggregate formation as well as disruption of the tissue structure. The zebrafish chst6 mutants generated here displayed human disease symptoms. A patent application (no 2022/021668**)** was filed, to use the mutant zebrafish as a preclinical model. This first in vivo MCD model reported here will be essential for drug discovery and efficacy testing as well as an understanding of the disease mechanism.

P280

Improving the safety of stem cell derived islet -based cell replacement therapy for type 1 diabetes via positron emission tomography

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Type 1 diabetes (T1D) results from destruction of insulin producing beta cells in the pancreatic islets. T1D can be cured with cell replacement therapy, by transplanting cadaveric human islets, but this is limited by shortage of donor islets and consequent need of systemic immunosuppression. Pluripotent stem cells can be differentiated in vitro into islets (SC-islets) in limitless quantities, and genome edited to become immune-evasive. SC-islet-based T1D cell replacement therapy is in clinical trials, where the first patient engrafted with SC-islets has become free of exogenous insulin injections, but another group reported occurrence of metastatic teratoma originating from supposed SC-islet material. Engineered immune evasion would increase aggressiveness of tumours originating from SC-islet grafts.

There is enormous promise, but great risks in SC-islet cell replacement therapy. However, methods to monitor the SC-islet grafts are inadequate, compromising safety, as measuring insulin secretion is influenced by blood glucose, graft volume and beta cell number and functional state. Our aim was to develop positron emission tomography (PET) enabling non-invasive monitoring of SC-islet graft volume independent of their functional state and composition. This would ensure their safety and allow optimisation of transplantation protocols for widespread clinical use.

We transplanted human SC-islets into calf-muscles of immunocompromised mice. Some mice were transplanted with different volume of SC-islets in each calf, to dissect the relationship between PET-tracer uptake and graft volume. Some mice were transplanted with wildtype SC-islets in one calf and hyper- or hypoactive SC-islets in the other, to study the relationship between uptake and graft functional state. These extremes of functional state were created by engineering LOF or GOF mutations, respectively, to the insulin-secretion-triggering K_{ATP}-channel gene *KCNJ11*. The mice were followed for 5 months and imaged with PET-tracers [^{18}F]-exendin and [^{18}F]-DOPA at multiple timepoints, followed by histological determination of the actual graft size and composition.

At the 5-mo timepoint, 90% of the grafts could be detected with [^{18}F]-exendin and 72% with [^{18}F]-DOPA, even though the smallest of them were < 1 mm^3 in actual volume. Importantly, the volume of grafts in PET correlated highly with their actual volume with both [^{18}F]-exendin (r^2=0.89) and [^{18}F]-DOPA (r^2=0.89). Longitudinal monitoring of the same graft with PET revealed different growth patterns for grafts of different purities. We then correlated PET-tracer uptake with graft composition. [^{18}F]-exendin concentration during PET correlated positively (r^2=0.43) with the percentage of beta cells in the graft, allowing estimation of their number noninvasively, impossible with current methods. Analyses correlating tracer uptake with additional aspects of graft composition e.g., percentage of undesired cells are ongoing. Graft functional state did not bias the uptake of either tracer in preliminary analyses.

In conclusion, PET-imaging allows sensitive detection of SC-islet grafts and quantification of their volume without being biased by functional status of the grafts. [^{18}F]-exendin had superior sensitivity than [^{18}F]-DOPA and allowed estimating the number of beta cells, but [^{18}F]-DOPA is

more widely available clinically. We propose monitoring SC-islet grafts in future clinical studies and T1D-patients with [^{18}F]-exendin and/or [^{18}F]-DOPA PET-imaging.

P281

Generation of a fully human pluripotent stem cell-derived model of blood-brain barrier for disease modelling

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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 vessels, 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 using specific transcription factors for each of these cell types recapitulating the developmental milestones of their differentiation lineage. To assemble the three cell types together we decided to generate assembloids that will recapitulate the spatial organization of the BBB both on transwell filters or aggregates freely floating into the medium. We demonstrated the generation of endothelial cell junctions by immunofluorescence for selective molecular markers. BBB functionality has been tested by TEER measurements and dye exclusion assays. The use of cell-identity transcription factors has a key role in improving the consistency of cell differentiation and subsequent maturation state. Co-culture of brain endothelium, mural cells and astrocytes produced a robust in vitro neurovascular model with long-lasting stability and functions. This defined differentiation system should broadly enable the use of human BBB endothelium for both disease modeling and biomedical applications.

P282

Towards a three-dimensional liver in vitro model for safety assessment in AAV-based gene therapy

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AAV vectors have become an important delivery method in Gene Therapy. Systemic administration of AAVs can lead to their accumulation not only in the desired location, but also in other organs. Due to its particular anatomy, the liver is prone to take up AAVs and it was observed in several clinical studies that the accumulation of AAVs may lead to severe liver toxicities and even fatalities. This raises significant concerns for AAV programs as the mechanisms of such AAV-driven liver toxicities are not understood. In vitro studies employ a variety of liver models, each with its own set of benefits and limitations, especially concerning the physiological relevance and availability of primary material. The use of human liver in vitro models could support the investigations of AAV-mediated liver toxicities and could be used to optimize AAVs in preclinical

studies. A liver model based on induced pluripotent stem cells (iPSC) could be a suitable tool because it could provide an unlimited supply of liver and liver-associated cells and recapitulate the in vivo scenario while transduced with AAVs. Therefore, we investigated the possibility of using iPS-derived hepatocyte organoids which were exposed to different AAV-GFP serotypes (AAV2, AAV8 and AAV9) and Moieties of Infection (MOI 10^4-10^6). Transduction efficacy of the individual AAVs was assessed by measuring the GFP signal using High Content Imaging Analysis over a culture period of 28 days. Our results showed serotype-dependent transduction efficiencies and revealed inter-donor variability. Of the three donors tested, AAV8 and AAV9 were more efficient than AAV2 in transducing iPS-derived hepatocytes with maximum levels of transduction after 21 days. Based on these preliminary results, we are aiming to establish an organoid liver organoid model with donor-matched iPS-derived hepatocytes, kupffer cells, liver sinusoidal endothelial cells and hepatic stellate cells, which will be used to investigate the potential mechanism of AAV-mediated liver toxicities.

P283

Transcriptomic profiling of iPS cell derived hepatocyte-like cells as genuine surrogates of primary liver hepatocytes

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Human induced pluripotent stem cells and their hepatocyte-like cell derivatives are promising to provide material for regenerative and toxicological assays. However, terminally differentiated HLCs have not been fully demonstrated as genuine surrogates of primary liver cells that share a significant liver gene expression profile. In this study, we perform in-depth transcriptomics that shows clear and significant differences between these cells, their precursor iPS cells and an array of normal and cancer cell types. We show by gene set enrichment analysis that genes critical for immune signalling pathways become downregulated upon differentiation revealing clearly dissimilar transcriptomics for each cell type under investigation. Our analysis found that HLCs exhibit a mild gene signature characteristic of acute lymphoblastic leukaemia but not other selected cancers and representative of primary hepatocytes making them genuinely valuable for surrogates of liver cells.

P284

In vivo model of Hutchinson-Gilford Progeria Syndrome to test new therapeutic strategies against 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 zebrafish 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.
The *zmpste24*sa9593 mutation line was imported into the CICA aquarium system from the EZRC. This line was generated at the Sanger Center (Cambridge, UK) by N-ethyl-nitrosurea-induced mutagenesis. We carried out crosses between the mutant line *zmpste24*sa9593 and wild lines of zebrafish to eliminate unwanted mutations, genotyping of the adult individuals of the new generations were performed to identify those that carry the mutation of interest. After there, we evaluated several genes in the zebrafish HGPS model, such markers of senescence (*p53*, *MDM2*, *LMNA*, *P18*, *p27 and CDKN2A/B*), oxidative stress and purine metabolism (*CD13*, *ENO* and *PRPS1*) using qPCR-RT. The senescence markers were statistically significant increase in our HGPS model *vs* wild type. In this study, we developed a zebrafish HGPS to study new therapeutic strategies to treat senescence in the future.

P285

Human induced pluripotent stem cell-derived models as a platform to advance AAV testing with high confidence

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AAV-based gene therapy is a rapidly growing clinical field that is revolutionizing the treatment of genetic diseases. Despite its potential, only a few drugs received market approval from regulators to date. One major concern in the field is the difficulty of translating results from bench to clinic and new approaches must be adopted to transform this scenario. Although (humanized) animal models provide invaluable insights for preclinical evaluation of gene therapies, their predictions do not match human responses to treatment due to interspecies differences. This has greatly contributed to the translation gap. To illustrate, selection of AAV serotypes and tropisms is crucial to develop a safe and effective gene therapy. However, there is clinical evidence showing that AAV serotypes and tropisms are not preserved among species.

In the past decade, human induced pluripotent stem cells (hiPSCs) have emerged as a powerful tool to bring the human biological context earlier into therapeutic development. These cells can self-renew and differentiate into functional cell types which recapitulate human pathophysiology, genetic diversity, and human serotype. Optimization and efficacy testing of AAVs in a relevant human context before moving into animal models can save time, reduce the number of animals needed and increase success in later stages.

Using hiPSC cells derived from Friedreich's ataxia (FRDA) patients-the most common hereditary ataxia- Ncardia manufactured a large batch (>500 million cells) of cardiomyocytes using a proprietary bioreactor based differentiation protocol. The generated FRDA hiPSC derived cardiomyocytes (hiPSC-CMs) showed cellular phenotypes consistent with diastolic dysfunction and oxidative stress effectively recapitulating *in vitro* the disease phenotype. A significantly longer

calcium transient duration in conjunction with a longer decay time was observed or FRDA hiPSC-CMs compared to control (healthy) hiPSC-CMs. Additionally, reactive oxygen species (ROS) levels evaluated by flow cytometry showed a significant increase in FRDA hiPSC-CMs. The efficacy of an AAV overexpressing frataxin (the target protein deficient in FRDA patients) was evaluated using this model. When the cardiomyocytes were transduced with the FXN AAV at an MOI of 10.000, calcium transient duration was significantly decreased in FRDA hiPSC-CMs and ROS levels were significantly reduced, returning to values comparable to the ones of healthy hiPSC-CMs.

With the effective rescue of the disease phenotype we now have a platform for testing and comparing the efficacy and cardiotoxicity of several AAV batches, that facilitates the selection of the most promising vectors early in the development process, saving time and resources.

P286

Developing and Characterizing a Murine Model with the Liver and Lung Pathology Seen in Human Alpha-1 Anti-Trypsin Deficiency Patients

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Alpha-1 Anti-Trypsin Deficiency (AATD) is characterized by lung disease due to a decrease in serum AAT levels, which subsequently allows neutrophil elastase to act largely unopposed in the pulmonary parenchyma. The mutation of the SERPINA1 gene causing the majority of AATD disease is caused by a point mutation (PiZ) that leads to accumulation of protein aggregates in the liver and subsequently decreased serum levels of AAT. This can result in either acute or chronic liver disease in a subset of patients as well as lung disease.

Previous murine models of AATD have either recapitulated the liver phenotype (PiZ expressing mice) or the lung phenotype (AAT-Null mouse) but not both. To model both disease phenotypes in one mouse we built upon previous work in our group creating the AAT-Null mouse. This mouse was created by using gene editing to knock-out all copies of the murine SERPINA1 gene (that encodes AAT). This resulted in a previously published model that develops emphysema with both aging and exposure to an acute inflammatory stimuli (Bacterial lipopolysaccharide (LPS)). We crossed this AAT-Null mouse with the previously available PiZ mouse, which expresses the human SERPINA1 gene with the PiZ point mutation.

We backcrossed these two strains to obtain a mouse model that expresses human PiZ AAT without any murine AAT expression. We then backcrossed those animals with C57Bl6 mice to create a homogeneous genetic background. To characterize these mice, we performed lung function testing with aging and after LPS challenge to determine if they develop emphysematous changes like the AAT-Null mouse model. We hypothesized that they may have less sensitivity to aging and inflammatory insults due to residual anti-neutrophil elastase capacity of the PiZ AAT that is able to be secreted from the liver. We found this to be true in the initial LPS challenge studies. The PiZ AAT-Null mice required increased dosage of LPS to develop the same pulmonary changes seen in the AAT-Null model.

To confirm the changes seen in the lung function parameters, we also performed histology of the lung to determine changes in the pulmonary parenchyma as well as inflammatory infiltrates. The

hepatic tissue was also assessed for accumulation of PiZ AAT aggregates. These aggregates proved to be similar to what was seen in the previous PiZ AAT mouse model, consistent with what we expected.

In addition to lung function testing and histologic analysis, we will also assess the anti-neutrophil elastase activity in comparison to age-matched AAT-Null mice. These results are pending.

In summary, we have created a PiZ-AAT mouse model that appears to be recapitulated both the lung and liver disease seen in AATD patients. This mouse will be invaluable to future pre-clinical gene therapy and gene editing studies.

P287

Generation of human hematopoietic stem cells with specific Fanconi anemia patient mutations

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New gene editing approaches are being developed to precisely correct the majority of the mutations accounting for different genetic disorders. To demonstrate the efficiency and safety of these editing tools to correct hematopoietic stem cells (HSCs) from patients with rare diseases, bone marrow samples from these patients are needed, something that frequently constitutes a bottleneck in the development of these therapies. In the particular case of Fanconi anemia (FA), an additional challenge is the limited content of HSCs in the bone marrow of these patients. To overcome these limitations and facilitate the evaluation of different gene editing approaches, $CD34^+$ cells from healthy donors were targeted with the CRISPR/Cas9 system and AAV6 vectors that carried a DNA sequence with the most prevalent *FANCA* mutation in Spain (*FANCA* c.295 C>T) as a donor template. Our results showed up to 65% substitution of the WT *FANCA* sequence by the c.295C>T *FANCA* mutation. Edited cells showed marked proliferative disadvantage, reduced clonogenic capacity and hypersensitivity to the inter-strand cross-linking agent, mitomycin C (MMC), thus resembling the characteristic phenotype of FA HSPCs. When edited cells were transplanted into immunodeficient mice, an 85% reduction of human hematopoietic engraftment was observed compared to engraftments of mock-edited HSPCs, also mimicking the poor repopulation ability of uncorrected FA HSCs. To generate FA-like HSCs harboring the c.1083+2T>C *FANCA* mutation an adenosine base editing approach was selected due to the high efficiency of this system to target HSPCs. In this case, up to 90% editing efficacy in HSPCs was observed. Once again, a decreased in vitro proliferation and MMC-hypersensitivity was associated with the insertion of the c.1083+2T>C *FANCA* mutation in healthy HSPCs. Taken together, our results show that gene editing allows the generation of FA-like HSPCs harboring specific mutations previously described in FA patients. The generation of disease-like HSPCs as surrogates of patient-derived HSPCs will facilitate the development of novel gene editing systems aiming the treatment of rare hematopoietic diseases, including FA.

P288

Unveiling the molecular basis of autism spectrum disorders: investigating genes of interest through brain organoids and zebrafish

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Autism Spectrum Disorders (ASD) represent a heterogeneous group of neurodevelopmental conditions characterized by impairments in socialization and communication, as well as the presence of restricted interests and repetitive behaviours. Both environmental and genetic factors are involved in ASD development, but despite extensive research, the exact causes and underlying mechanisms of ASD remain largely unknown. The emergence of Next-Generation Sequencing (NGS) technologies has significantly advanced our ability to identify *de novo* genomic variants in individuals with ASD. However, up to date, most of these variants lack functional validation that supports these findings.

In this communication, our focus lies on the generation of *in vitro* and *in vivo* models to study the role of novel genes that have been linked to ASD through NGS analysis. This approach aims to improve our understanding of the molecular basis of the disorder, to improve the genetic counselling provided to the families, and to identify new potential therapeutic targets.

Our *in vitro* model relies on the use of human induced pluripotent stem cells (hiPSCs), due to their remarkable capability to differentiate into neuronal-like cell lines. Utilizing CRISPR/Cas9 technology, we selectively knockout target genes in these cells and extensively characterize the resulting edited hiPSCs. After that, the knockout cell lines and their isogenic controls were differentiated into suitable models to study neurodevelopment, including cortical-like neuronal cultures (2D) and dorsal forebrain organoids (3D). Through comprehensive analysis at various time points, we aim to identify potential abnormalities in the differentiation process induced by gene alterations. To achieve this, we employ techniques such as immunostaining, RT-qPCR, calcium imaging, and single-cell RNA sequencing (scRNA-seq) to gain comprehensive insights into the functional consequences of gene perturbations.

Recognizing the inherent limitations of *in vitro* models in fully recapitulating the complexity of ASD phenotypes, we extend our investigations to *in vivo* models to explore behavioural and morphological alterations associated with ASD. Our initial approach involved transient silencing of target genes using morpholinos in zebrafish, a well-established animal model in neuroscience. Furthermore, we generated stable mutant zebrafish lines to enable more in-depth and long-term analysis. By meticulously characterizing and comparing homozygous and heterozygous individuals with wild-type counterparts, we aimed to identify morphological, transcriptional, and behavioural changes associated with mutations in our genes of interest.

Through the integration of *in vitro* and *in vivo* models, our study seeks to unravel the functional implications of *de novo* genomic variants in the development of ASD. By shedding light on the underlying mechanisms, we aspire to contribute to our understanding of the disorder, provide

valuable insights for genetic counselling, and pave the way for the identification of potential therapeutic targets. Ultimately, our research endeavours to make significant strides in addressing the complex challenges posed by ASD and improving the lives of individuals and families affected by this condition.

P289

Evaluating the transduction efficiency of different AAV serotypes on hiPSC-derived cardiomyocytes

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AAV-based gene therapy is a rapidly growing clinical field that is revolutionizing the treatment of genetic diseases. Despite its potential, only a few drugs received market approval from regulators to date. One major concern in the field is the difficulty of translating results from bench to clinic and new approaches must be adopted to transform this scenario. Although (humanized) animal models provide invaluable insights for preclinical evaluation of gene therapies, their predictions do not match human responses to treatment due to interspecies differences. This has greatly contributed to the translation gap. To illustrate, selection of AAV serotypes and tropisms is crucial to develop a safe and effective gene therapy. However, there is clinical evidence showing that AAV serotypes and tropisms are not preserved among species.

In the past decade, human induced pluripotent stem cells (hiPSCs) have emerged as a powerful tool to bring the human biological context earlier into therapeutic development. These cells can self-renew and differentiate into functional cell types which recapitulate human pathophysiology, genetic diversity, and human serotype.

In order to evaluate AAV transduction efficiency in hiPSC derived cardiomyocyte cells (hiPSC-CMs) a panel of AAVs of different serotypes, all expressing GFP, was tested at several MOI values in our bioreactor generated (healthy control) hiPSC-CMs called Ncytes. Fluorescence intensity levels for GFP were recorded daily for a period of 10 days post transduction using a microplate reader. At the final experimental timepoint the nuclei of the transduced hiPSC-CMs were stained with Hoechst dye and the cells were imaged using a high content image confocal microscope. The parameters of cell %viability (number of nuclei/average number of nuclei of vehicle control), %transduction efficiency (number of GFP+ nuclei/number of total nuclei) and average fluorescence intensity were analyzed for all conditions.

Most AAV serotypes at their respective MOIs, had an acceptable GFP signal (2-7x fluorescence intensity above local background). The only vector that failed to transduce hiPSC-CMs was AAV4. For all other vectors transduction efficiency increased with increasing MOIs as expected. The most efficient transductions were achieved using AAV1 and AAV5 vectors. Cytotoxicity was observed at higher MOIs (>10.000) for several vectors.

Using our high content imager we now have a suitable platform to evaluate the transduction efficiency and cytotoxicity of test AAVs. This not only helps us to identify which AAV serotypes are most suitable for transducing our Ncyte cells but also allows us to assess the transduction potential of AAVs supplied by our clients cost effectively, prior testing their efficacy in downstream functional assays.

P290

Developing a dual-reporter iPSC-line using genetically encoded voltage and calcium indicators (GEVI/GECI)

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Genetically encoded indicators are a novel tool that allow electrophysiological analysis of electrically active cells in a high-throughput and non-invasive manner. In this technique cells are transfected with constructs containing a GEVI and/or a GECI. Once these genes are expressed, a flux in fluorescence intensity can be recorded with a fluorescence microscope when the cell fires an action potential or when a calcium flux takes place inside the cell. Our goal is to select a GEVI and a GECI with suitable characteristics for use in hiPSC-derived cardiomyocytes (hiPSC-CM) and develop a dual-reporter hiPSC-line containing both this GEVI and GECI inserted into the genome using CRISPR/Cas9. This line, possessing stable expression of both indicators, can then be differentiated towards hiPSC-CM that can be used to create disease models and study e.g. genetic variants associated with inherited cardiac arrhythmias. Based on literature, we selected a list of five GEVIs and two GECIs to first test in our own in-house differentiated control hiPSC-CM. Plasmids were ordered through Addgene and AAV particles were made from plasmids containing AAV2 ITRs. Briefly, AAV2 ITR containing plasmids were co-transfected together with pAAV-DJ and pHelper plasmids into HEK293T cells and after 72 hours, medium and cells are collected and AAV particles are purified by freezing/thawing and by centrifugation. Subsequently, AAV transduction and/or lipofection were used to bring these GEVI/GECIs into the hiPSC-CMs seeded in glass-like bottom 96-well plates, followed by spinning disc live confocal microscopy of the transfected cells to screen and select the best indicators for calcium and voltage imaging. Lipofections and transductions were performed in duplo. Three different volumes of AAV suspension (0.5, 1 and 10µL) were used for transduction. Lipofections were performed using the Lipofectamine 3000 kit. Multiple wells were not transfected in order to monitor the activity of the hiPSC-CM. Analysis of the recordings was performed using ImageJ and R. We obtained the best results with the GECI NCaMP7 and the GEVI paQuasAr3 after AAV transduction of the hiPSC-CMs and successfully imaged calcium kinetics and action potentials, respectively. Future steps include optimizing the transfection procedure and the imaging parameters, co-transfection of the hiPSC-CM with both the GEVI and the GECI and ultimately introducing these indicators into a control hiPSC-line using CRISPR/Cas9.

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Neural induction and neuronal maturation of induced pluripotent stem cell is compromised in Machado Joseph-Disease lines

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Machado-Joseph disease (MJD), also known as Spinocerebellar Ataxia Type 3 (SCA3), is a polyglutamine disease and the most common of the dominantly inherited ataxias worldwide. MJD/SCA3 is caused by the over-repetition of a CAG trinucleotide in the *ATXN3* gene, which translates into an expanded polyglutamine tract within the protein ataxin-3. The mutant ataxin-3 protein becomes prone to misfolding, aggregates in neurons, leading to cell dysfunction and death, particularly in the cerebellum. As such, the main clinical hallmark presented by these patients is progressive ataxia. MJD/SCA3 is very disable and causes premature death, but up to date there is no effective therapy. Disease-specific induced pluripotent stem cells (iPSCs) have become leading tools for developing physiologically-relevant and predictive disease models for which there is an urgent need in MJD/SCA3 preclinical studies.

Here, we investigated the neural induction efficiency and neuronal maturation in iPSCs derived from MJD/SCA3 patients compared to iPSCs derived from healthy individuals (controls). Control and MJD/SCA3 iPSCs lines were submitted to a dual SMAD inhibition monolayer protocol and the cell plating density, cell expansion, cell viability and cell morphology were evaluated during neural induction. Neural progenitor cells (NPCs) obtained were characterized by the analysis of neural progenitor cell and early neuron markers (PAX6, SOX1, TUJ1). NPCs were further differentiated and the % of NeuN positive cells (mature neurons) was determined. During neural induction, we observed a reduction in cell expansion in MJD/SCA3 lines compared to controls. A higher cell plating density was used to obtain NPCs from MJD/SCA3 iPSC lines. Moreover, while during neural induction of control lines we observed an increase in cell viability, this was not observed for MJD/SCA3 cell lines. We then calculated the efficiency of neural induction by determining the number of the successful neural induction protocols from the overall protocols performed for each cell line. An 82% efficiency was observed for control lines, in opposition to the 17% neural induction efficiency obtained for MJD/SCA3 cell lines. Nevertheless, when comparing NPCs obtained from successful protocols of both MJD/SCA3 and control lines no differences were observed in the levels of SOX1, PAX6 and TUJ1. Finally, upon NPCs differentiation into mature neurons, a lower percentage of NeuN positive cells were observed in MJD/SCA3 compared to controls. All together these data suggest that there is an impairment in neural induction and neuronal maturation in MJD/SCA3. A depth understanding of associated pathological mechanisms are currently being investigated. These iPSCs derived neuronal models might be used as powerful tools for a deep understanding of disease pathogenesis, discovery of biomarkers, and in pre-clinical studies for the development of effective therapies for MJD/SCA3.

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In vitro Human cell-based 3D Engineered Muscle Tissues Show Clinically-Relevant Functional Markers of Duchenne Muscular Dystrophy Model

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Accurately modelling healthy and disease conditions in vitro is vital for understanding the potential safety and efficacy of new therapeutics before they are given to patients. For cardiac and skeletal muscle diseases, direct assessment of muscle contraction is a reliable metric to study overall tissue function, as other 'proxy' measurements, such as cell diameter, are poor predictors of muscle strength. Human 3D engineered muscle tissues (EMTs) from induced pluripotent stem cell and primary cell sources hold great potential for modelling contractile function. However, the complex bioengineering strategies required to generate reproducible and predictive models

presents limitations for many investigators. Here, we have developed a turnkey platform for facile fabrication of 3D muscle constructs in concert with label-free, push-button measurements of contractility. Tissues made on the platform are highly reproducible and can be made with virtually any cell source. The platform features individual, well-based control of stimulation and parallel direct measurement of contractility simultaneously across 24 tissues with little to no user input. This approach enables clinically relevant functional measurements of muscle, stratification of healthy and diseased muscle phenotypes, and facilitates therapeutic modality-agnostic, dose-dependent compound safety and efficacy screening.

Here, we present a 3D model of Duchenne muscular dystrophy that utilizes skeletal muscle EMTs formed from a commercially-available isogenic pair of healthy and diseased human cells. These cryopreserved cells are optimized for casting directly into 3D organoids and designed to minimize the variability-producing steps of cell expansion and passaging that are required to produce sufficient cell numbers for generating 3D constructs. These tissues achieve robust twitch and tetanic responses upon stimulation. The model demonstrates a host of easily measured and clinically-relevant functional deficits across numerous metrics of contractility, including force and fatigability. Tissues remain functional for months in culture, providing a significant experimental window to study both therapeutic effect and disease phenotypes that may present at later stages of development and maturity. Increased levels of adult MyHC isoforms suggest enriched maturation, providing a more physiologically relevant human model. In addition, we also stretch these tissues under load to model eccentric contractions and muscle injury. We show increased levels of creatine kinase, a biomarker of cellular damage, in the tissue medium. Finally, we have developed a method to preserve living EMTs in a biocompatible gel that permits transfer between labs under ambient storage conditions. Tissues remain viable and fully functional upon gel dissolution for direct interrogation with therapeutic compounds, which enables the production of tissues from a single source, further enhancing reproducibility, reliability, and drastically lowering cost.

These data demonstrate a first-and-only commercial platform integrating individual, well-based control of electrical stimulation across a 24-well plate to pace 3D tissues, modeling exercise regimens or damage protocols in muscle constructs. Eccentric contraction modeling is achieved by stretching 3D muscle organoids under load, exacerbating the DMD phenotype and presenting a challenge for gene therapy constructs targeting injury prevention in this disease. Stimulation is coupled with automated assessment of 3D muscle contraction, providing an inclusive, semi-high-throughput platform for disease modeling, gene therapy, and drug discovery.

P293

Brain organoids as a platform to study subcellular trafficking of recombinant AAV vectors

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Adeno-associated virus (AAV) is the leading gene therapy vector in clinical development. However, efficacious vector delivery to target cells remains a challenge. The subcellular bottlenecks underlying suboptimal transduction remain elusive, partly due to the lack of knowledge about the complex subcellular trafficking process of AAV. Findings on preferred trafficking pathways, endosomal escape, nuclear entry, viral genome release and dynamics of these processes are mostly obtained in adherent cell lines in fixed samples. These data are often ambiguous and might be less representative of real-time trafficking of vectors in human tissues.

In this study, we explore if human organoids more closely mimicking human tissues, represent a relevant model to study transduction and subcellular trafficking of recombinant AAV vectors.

We have successfully generated human induced pluripotent stem cell-derived brain organoids, which display characteristic hallmarks of human brain development as determined by immunostaining. Importantly, we demonstrate that neurotropic AAV capsid variants, which cannot bind heparan sulphate proteoglycan and show poor transduction in 2D primary cell cultures, have the ability to transduce neurons in these brain organoids. These data suggest that brain organoids provide similar transduction profiles as observed *in vivo*.

In preparation for vector trafficking studies, we set up a confocal (1-photon/multi-photon) microscopy imaging strategy, which enables live imaging of AAV trafficking in transduced 2D cell lines and intact brain organoids. We also developed an immunofluoresence-FISH assay in human fibroblast cells in order to visualize viral genome release. We will present data on the dynamics of trafficking of different neurotropic AAV vectors. Comparing AAV trafficking data in 2D cells (HeLa, hiPSC) and 3D organoids, in combination with staining for certain cell types and/or markers will provide insight into the key parameters of *in vivo* transduction. This novel platform opens up the possibility to screen for capsids which efficaciously traffic and translocate into the nucleus.

P294

Allele-specific targeting of mutant DMPK by antisense oligonucleotides in DM1 patient iPSC

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Myotonic dystrophy type 1 (DM1), a dominant hereditary muscular dystrophy, is caused by an abnormal expansion of a (CTG)n trinucleotide repeat in the 3′ UTR of the human dystrophia myotonica protein kinase (DMPK) gene. A leading approach to treat DM1 uses DMPK-targeting antisense oligonucleotides (ASOs) to reduce levels of toxic RNA. However, complete DMPK gene antisense knockdown may cause unknown risks. Here we established a screening platform with patients and control induced pluripotent stem cells to test antisense oligonucleotides (ASOs) for their effects on DMPK expression. First, we screened pairs of ASOs designed to selectively target the mutant or the wild-type allele by taking advantage of an SNP (rs527221) in DMPK that is present in 15% of humans. Then, we suppressed mutant and wild-type DMPK levels by >90% after a singular treatment. To reduce cytotoxicity, we found ASOmut to reduce levels of DMPK by 25% when we used 100nM low concentration. Next, we will test this ASO in a long-term study for about 1 week. This study proves that allele-specific DMPK lowering by selective ASOs is feasible in a DMPK patient's human cell culture model.

P295

Analysis of exosomes derived from human umbilical cord mesenchymal stromal cells expanded in vitro in xeno-free conditions.

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Mesenchymal stromal cells (MSCs) are used in cell therapy due to their ability to modulate the microenvironment through the secretion of bioactive molecules that possess immunomodulatory and trophic (regenerative) properties. This treatment exhibits anti-inflammatory, paracrine, and autocrine trophic effects, interacting with local cells, and various actions, such as anti-apoptotic, anti-fibrotic, angiogenic, chemoattractant, reparative, and regenerative have been reported. To date, the analysis of the paracrine activity of MSCs is gaining significance. Recognizing the characteristics of extracellular vesicles and their potential is crucial during the in vitro expansion of MSCs. The objective of this study was to characterize the extracellular vesicles/exosomes (EV's/L) obtained from an in vitro culture supplemented with human platelet lysate (hPL) after passage 5.

Wharton's jelly (WJ)-derived MSCs from the umbilical cord were expanded in culture medium supplemented with 10% hPL until passage 5. Once the cell culture reached 80% confluency, the supplemented medium was removed and replaced by DMEM without supplementation. The cells were incubated for 24 hours and after this time the culture medium was collected to perform a purification of the exosomes by diafiltration. The exosomes obtained were analyzed by flow cytometry and electron microscopy. Finally, a proteomic characterization of the exosomes obtained was carried out.

Under the evaluated conditions, 1.2x10^12 - 1.7x10^12 EV's/L were obtained, and the proteomic analysis showed up to 34 exosome-exclusive proteins. Electron microscopy analysis revealed the size of the isolated exosomes, with ranges between 80-240 nm. Extracellular vesicles markers expression was confirmed, with CD9 = 25.1%, CD63 = 71.5% and CD81 = 20.4%.1966 different proteins were identified through ExoCarta database, and classified according to molecular functions in binding, catalytic activity, and regulation proteins; according to biological processes in interaction between cells, and cellular regulation proteins; and according to signaling pathways in proteins related to growth factors: EGF, FGF, IGF, PDGF, TGFB, VEGF.

Overall, these results suggest that the presence of a significant number of exosome-exclusive proteins qualifies the samples as highly enriched, and the functions attributed to the characterized exosomes indicate their involvement in various biological pathways, thus highlighting their therapeutic potential.

P296

Exploring the role of genomic variants in rare neurodevelopmental disorders using hiPSCs and brain organoids: implications for advanced therapeutic strategies

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Rare neurodevelopmental disorders, such as Lafora Disease and *GABRB3*-related syndromes, pose significant challenges due to their rarity and complexity, such as the understanding of their underlying molecular aetiology, hindering the development of targeted therapies. The use of human induced pluripotent stem cells (hiPSCs) and genome editing technologies presents a promising approach to investigate the role of genomic variants in these disorders.

Herein, we generated hiPSC lines presenting disease-associated mutations identified in patients with rare neurodevelopmental disorders, namely Lafora Disease and *GABRB3*-related syndromes -knock-in models-, and also other cell lines with mutations in disease-related genes -knock-down and knock-out models-, using CRISPR-Cas9 genome editing technology.
Lafora Disease stands as a rare autosomal recessive disorder characterised by the accumulation of insoluble intracellular glycogen-like particles known as Lafora bodies. In order to model this disease, hiPSCs were edited introducing causing mutations in *EPM2A* and *NHLRC1* genes. Regarding *GABRB3*-related syndromes, a group of rare neurodevelopmental disorders characterised by intellectual disability, speech and language impairment, and epilepsy, we generated hiPSCs with mutations in the *GABRB3* gene.

Our next on-going step is to differentiate these genetically modified hiPSCs into cell types affected by the disorders, such as those in the central nervous system (CNS). With that aim, we are using our optimised differentiation protocols to obtain two-dimensional (2D) and three-dimensional (3D) models such as brain organoids, that closely resemble the architecture and function of the human brain. In parallel, we are also differentiating hiPSCs derived from patients into the same cell types affected by the disorders to compare them with our models and establish the significance of genomic variants.

Our current focus is on producing 2D models of astrocytes and neurons for Lafora disease, as these are the primary cell types affected by the disorder. Similarly, for *GABRB3*-related syndromes, we are generating 2D models of cortical neurons, as these cells play a crucial role in cognitive function and are impacted in these disorders. Additionally, we are differentiating both disease models and patient-derived hiPSCs into brain organoids.

Our hiPSC-based models provide a valuable platform to investigate the role of ultra-rare and variants of uncertain significance (VUS) in rare neurodevelopmental disorders. Our findings from comparing our models with hiPSCs from patients have the potential to improve genetic counselling, and accelerate the development of treatments for these debilitating and often fatal disorders. Utilising hiPSCs and brain organoids to investigate rare neurodevelopmental

disorders can enhance our understanding of their molecular mechanisms and aid in the development of targeted therapies for patients in the future.

Furthermore, we aim to utilise differentiated patient-derived hiPSCs to identify and screen therapeutic targets for advanced therapies, including genome editing and mRNA delivery.

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Engineering isogenic neuronal models of *ATXN3* knockout in Machado-Joseph Disease patient cells by CRISPR/Cas9

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Machado-Joseph disease (MJD), or Spinocerebellar Ataxia type 3 (SCA3), is a fatal inherited neurodegenerative disorder that arises from an aberrant expansion of the cytosine-adenine-guanine (CAG) trinucleotide within the coding region of the *ATXN3* gene. This results in the production of a mutant Ataxin-3 (ATXN3) protein harboring an extended polyglutamine (PolyQ) tract. The mutant ATXN3 is prone to aggregation leading to the formation of intranuclear inclusions and generation of toxic species which compromise neuronal viability, particularly in cerebellum. Consequently, MJD patients manifest clinical hallmarks characterized by progressive ataxia. To this date there is still no treatment for MJD. Major efforts to find a therapy have focused on post-transcriptional silencing of *ATXN3* by RNA interference to prevent the formation of ATXN3 protein. Numerous mechanisms have been connected to ATXN3 activity, from transcription to proteasomal regulation, and thus long-term consequences of its abrogation remains elusive. Moreover, no study has thoroughly investigated the impact of silencing *ATXN3* within a relevant human context.

To address this, we used CRISPR/Cas9 genome engineering to create isogenic lines in MJD patient-derived pluripotent stem cells (iPSC), that were then subjected to a differentiation protocol to generate a human neuronal model of *ATXN3* knockout.

For this purpose, we directed a Cas9 endonuclease to an early exon of *ATXN3* to promote gene silencing. Editing was confirmed by Sanger sequencing. Following single-cell clone screening, we obtained four distinct lines with i) non-edited, ii) hemizygous knockout of wild-type allele, iii) hemizygous knockout of mutant allele or iv) homozygous knockout of both *ATXN3* alleles. Each genotype was validated by western blot and Sanger sequencing. To evaluate the effect of each genotype on neuronal development, one clone per genotype was differentiated into neural cultures using two different differentiation methods: a conventional neural induction to form neural progenitor cells (NPCs) which are matured into a heterogenous neuron and glia population; and a rapid Neurogenin-mediated directed differentiation into homogenous neuronal cultures. In the first approach, we detected the presence of NPC markers PAX6, NESTIN, and SOX1 in NPCs but not in matured neural cultures. In these, we detected both GFAP-positive cells and cells expressing MAP2, GABA, and TUJ1 markers. In the second approach, we were able to bypass NPC formation and rapidly obtain neuronal cultures within 4 days. These cells were positive for the neuronal markers MAP2 and Beta III tubulin, with no GFAP expressing cells.

Evaluation of ATXN3-associated cell mechanisms, such as mitochondrial function, autophagy, proteasomal function, as well as global transcriptomic analysis are currently under investigation. Overall, these models will allow us to thoroughly investigate the contribution of ATXN3 toxic gain *versus* loss of function to MJD progression but also the impact of *ATXN3* silencing for potential treatment avenues.

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Scalable Platform for Large-Scale Production of Human iPSCs using an Automated Stirred Tank Bioreactor System for Bioprinting Applications

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Human induced pluripotent stem cells (hiPSCs) have emerged as a critical and versatile cell source in tissue engineering, enabling the generation of diverse and functional tissues for transplantation therapy and disease modeling. To achieve organ-scale tissue engineering, the production of billions of human cells, particularly wholly cellular bioinks for bioprinting applications, is crucial. However, conventional 2D cell culture methods are not easily scalable due to cost, space, and handling constraints. In this study, we successfully optimized the suspension culture of hiPSC-derived aggregates (hAs) using an automated 250 mL stirred tank bioreactor system. Furthermore, we demonstrated the scalability of these optimized parameters to an automated 1 L stirred tank bioreactor system.

Through a combination of a Design of Experiments approach and multivariate data analysis, we identified an optimal culture condition from a dataset of 15 conditions, varying impeller speeds, seeding density, and the use of polyvinyl alcohol. Optimal aggregate sizes and diameters were obtained by varying the impeller speed. Cell yield (1.8 – 2.3 E6 cells over 3 days), aggregate morphology (more than 72% for SCVI-15-derived hAs and 90% for WTC-11 derived hAs with diameters ≤ 300 µm, with an overall circularity of ≥ 0.73), and pluripotency marker expression (above 90% for OCT4, NANOG, SSEA-4, and TRA-1-60) were maintained over three serial passages in two distinct cell lines, SCVI-15 and WTC-11 cells. Additionally, our 4-day culture in a 1L bioreactor system resulted in a 16.6 – 20.4-fold cell expansion, generating approximately 4 billion cells per vessel while retaining >94% expression of pluripotency markers. The pluripotent aggregates can be subsequently differentiated into derivatives of the three germ layers, including cardiac aggregates, vascular, cortical, and intestinal organoids. The aggregates were compacted into a wholly cellular bioinks for rheological analysis and 3D bioprinting. The bioprinted tissues have high post-printing viability and can be differentiated into vascular and neuronal tissue. This study demonstrates an optimized suspension culture-to-3D bioprinting workflow using an automated stirred tank bioreactor system, enabling a more sustainable approach to billion cell-scale organ engineering. In the next phase, our focus will be dedicated to addressing the large-scale challenges by implementing a 10L-scale stirred tank bioreactor system.

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Endothelial cytoskeleton rearrangement and glycocalyx shortening in relation to HNF1A-MODY

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Maturity Onset Diabetes of the Young (MODY) is a monogenic hereditary form of diabetes. One of the most common MODY subtypes is associated with mutations in the hepatocyte nuclear factor 1-alpha (*HNF1A*) gene. HNF1A-MODY patients often develop endothelial dysfunction (ED) leading to vascular complications, however, the molecular basis of these changes is still unknown. In our previous work, we have shown that endothelial cells (ECs) with mutations in the *HNF1A* have increased vascular permeability after cytokine stimulation. To reveal the potential molecular pathways underlying this increased permeability, two sets of human induced pluripotent stem cells (hiPSCs) lines were used in the current study. The patient set consisted of four hiPSCs lines with two healthy/control lines and two hiPSCs lines derived from HNF1A-MODY patients. The isogenic set has a control (healthy) and two CRISPR/Cas9-mutated hiPSCs lines, with monoallelic or biallelic mutations in the *HNF1A*. All these lines were subsequently differentiated toward ECs (hiPSC-ECs) and used for further analysis. The global transcriptome analysis of the isogenic set of lines showed that cells with mutations in the *HNF1A* had differential expression of genes related to proteoglycans, actin-based cytoskeleton regulation, and several signalling pathways. Alteration of the actin-cytoskeleton in the mutated cells was further confirmed by a global proteome analysis. These changes phenotypically were presented by shorter glycocalyx and diminished presence of sialic acid and heparan sulphate in both isogenic and patient-specific hiPSC-ECs with mutations in the *HNF1A* as compared to the appropriate control cells. Moreover, *HNF1A*-mutated isogenic lines had increased migratory potential, which could be recapitulated also in primary ECs with silenced *HNF1A* expression. This was in line with a reduction in the observed actin stress fibres of the mutated cells. Taken together, these results reveal for the first time that mutations in the *HNF1A*, affect the endothelial function, through reduction of the glycocalyx layer and increase in the cell migration. All of the observed changes could account for increased vascular permeability, which could further contribute to the non-hyperglycaemia-related ED in HNF1A-MODY patients.

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P300

Towards human innate immune-competent 3D hepatic models to address early tissue response to therapeutic AAV vectors

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Recombinant adeno-associated viral vectors (rAAVs) are a platform of choice for gene therapy. However, liver-directed transduction with rAAV has been hindered by unpredicted immune responses mediated by neutralizing antibodies (humoral immunity) and CD8+ T cells (adaptive immunity), ultimately hampering long-term expression of the transgene. Animal models have been useful tools in addressing the humoral response but have failed to fully reproduce the adaptive immunity induced by rAAVs. In fact, the innate immune response that precedes the T-cell cytotoxicity in humans remains largely unknown; a report in a human cell model suggested a TLR2-mediated response by the liver non-parenchymal cells (NPCs). Therefore, to address the innate response, human cell models recapitulative of the NPC compartments and of the liver microenvironment are required.

Herein, the aim is to develop a human 3D model to investigate the contribution of the different liver cell compartments to the innate immune response to rAAVs. Employing a step-by-step reconstruction strategy, we started by incorporating human hepatocytes, the therapeutic target cells, and Kupffer cells (KCs), the liver resident macrophages. KCs are the main mediators of early innate responses, can act as non-professional antigen presenting cells and elicit T cell activation. Upon infection or injury, the KC niche is replenished via recruitment and differentiation of circulating monocytes (Mo). We hypothesized that co-culturing human Mo isolated from peripheral blood mononuclear cells with hepatocytes would recapitulate KC differentiation. Therefore, we developed a Kupffer-like cell (KLC) differentiation protocol, leveraging from an in-house method for long-term culture of PHH spheroid. We started by screening PHHs from different donors for: aggregation capacity, with maintenance of hepatocyte identity, polarization, and biosynthetic function (assessed by protein detection of HNF4α, ZO-1, Collagen IV and albumin); and rAAV transduction efficiency (assessed by transgene expression). Co-culture parameters (e.g., medium composition, cell ratio, differentiation time) were optimized to attain efficient differentiation of Mo into KLCs, while retaining high PHH viability and identity. Co-culture of Mo with spheroids of PHH from three distinct donors induced upregulation of KC-associated genes (e.g., MARCO, VSIG4, CD163 and CD16), concomitant with detection of the respective proteins in KLCs. Functional assessment of KLCs within 3D co-cultures is ongoing, employing prototypical immune modulators. As a proof-of-concept of gene therapy applications, the co-cultures were transduced with rAAV expressing fluorescent transgenes. The experimental conditions for transduction (MOI) and time of detection of transgene were selected from a first screen in PHH spheroids. Differentiated 3D co-cultures mounted a response as early as 6h post-transduction, with de novo expression of at least one inflammatory cytokine (e.g., IL-6, IL-8).

Our data suggests the suitability of the novel model to address KC response within a human liver microenvironment. Incorporation of additional NPCs implicated in innate immune responses will increase the biological relevance of the model and widen its application in addressing the liver early response to rAAV and other ATMPs, as well as a tool for preclinical assessment of such therapies.

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P302

Early safety and efficacy observations following the first use of TSHA-102 gene therapy in a patient with Rett Syndrome

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The REVEAL Adult study (NCT05606614), a first-in-human trial of TSHA-102 gene therapy, is actively enrolling women ≥18 years old with Rett Syndrome (RS). The first enrolled participant received TSHA-102 treatment with immunoprophylaxis at the Centre Hospitalier Universitaire Sainte-Justine (Montréal, Canada). As specified in the study protocol, her case was reviewed by the Independent Data Monitoring Committee (IDMC) on Day 42 post-treatment.

Participant 1 is a 20-yo woman diagnosed with RS at age 3. She presented with global delay, followed by slow regression of skills. She could sit at 6 months but never crawled or walked independently or developed a pincer grasp. She learned a few words from 11 months onwards, which she eventually lost. Following speech and hand-use regression, stereotypic hand movement developed after age 2. She has been unable to stand without support since age 6 and stopped reaching or grasping around age 7. Seizures, developing at age 3, proved refractory to multiple medications. They are generally well-controlled with clobazam and phenytoin, despite occasional breakthrough seizures during viral infections or when her blood phenytoin declines to <100 mmol/L. Ongoing medical challenges include persistent constipation, recurrent pneumonia, scoliosis, osteopenia, and a benign thyroid nodule identified in January 2023.

Following sirolimus and prednisolone initiation at Day -7, TSHA-102 was injected intrathecally on Day 0. The participant experienced a clinically nonsignificant blood pressure decline, resolving within 8 hours. Oximetry following treatment showed stable tissue perfusion. Treatment-related adverse events included: an episode of mild (Grade 1) pyrexia on Day +1 (deemed TSHA-102-related), managed with acetaminophen and resolving in <24 hours; and mild irritability starting on Day +5 (prednisolone-related). Post-treatment hematology and other labs were unremarkable. There were no serious adverse events, irrespective of causality.

Starting Week 2 after TSHA-102 treatment, caregiver reports and key efficacy metrics showed clinical improvement. Week 4 clinical global impression change scores rated by physicians (CGI_i) and caregivers (PGI_i) were rated at 2 (“Much improved”) and 3 (“A little better”), respectively. RS Behavior Questionnaire (RSBQ) total score showed a 23-point improvement over baseline; of the 8 RSBQ subscales, 6 showed substantial improvement, including “Hand behaviors” (declining from 12 to 5 points). The “Night-time behaviors” score declined from 3 to 0 points, consistent with caregiver reports that the patient was sleeping peacefully all night, for the first time in their

memory. Conversely, there were no significant changes observed on the Revised Motor Behavior Assessment (R-MBA) or any subscales. However, the participant started being able to sit unassisted for a few minutes by Day 35. Finally, whereas two seizures were recorded in the 10-day run-up to therapy, none occurred between Days 0 and +35, despite low blood phenytoin.

Because Participant 1 experienced no dose-limiting toxicities, and considering the rapid clinical benefit she appears to be experiencing, the IDMC approved continuation of the REVEAL Adult study, with Participants 2 and 3 to receive the same dose of TSHA-102 as Participant 1, later in this calendar year.

P303

Genome Therapy: no access without acceptance

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Discussion about accessibility to novel genome modulating techniques such as CRISPR/Cas rests on a crucial precondition: public acceptance of this technology. As several gene therapy trials carried out in the past have resulted in disastrous outcomes, new genetic biotechnologies are eyed with considerable mistrust by the public, which is why attention to these public attitudes is crucial to the issue of accessibility. This task starts with the question of how to correctly conceptualize the issue of novel genome modulating techniques and genomic therapies for a public debate - and what went wrong so far.

Currently, public perception of new technologies is particularly shaped by metaphors and analogies. Especially in the communication of genetics and genomics research, talk of “information”, “code”, “letter”, or “book” has become pervasive. It is from these linguistic metaphors that the metaphors for CRISPR-based genetic technologies are derived: Scientists call CRISPR an "editing" or "rewriting" tool. It would *be* a "genome scissors", a "molecular scalpel" or a "genetic word processor". While metaphors can be useful in exemplifying a biotechnology to a certain extent, they here entail a problematic ontologization: CRISPR/Cas is given a certain reality that it does not actually possess, because it promises unprecedented precision and the ability to manipulate the genome at will. These metaphors not only fail to adequately describe the physical mechanisms underlying the technique, but also conceal the complexity of the genome as the target of CRISPR/Cas, thus downplaying risks of unwanted “off-target-effects”. Moreover, comparisons with word processors may raise the expectation that deletions can be undone.

To achieve adequate communication about CRISPR/Cas, we need to find ways of informing the public about the risks and uncertainties of the technology, taking into account the state of current post-genomic debates. The predominant linguistic representations of CRISPR/Cas and the genome ignore the central philosophical, scientific and ethical debates in post-genomic research, and thus miss the opportunity for reflective public discourse regarding the issue of novel genomic therapies. For instance, scientific developments have shown that multiple cellular, genetic and epigenetic processes are equally causally necessary to determine a developmental outcome. This fact, dubbed “causal democracy”, that a large number of factors may be responsible for a variety of developmental and regulatory activities ultimately leading to a particular trait or disease, not least entails significant impediments to the screening of unintended consequences of CRISPR editing.

To promote future acceptance of - and thus *access* to - CRISPR/Cas-mediated gene modification and genomic therapies, an “informed” public is needed. In order to achieve this goal, we propose three steps involving different perspectives on the problem areas outlined: First, we will analyze

the metaphors used in the context of CRISPR-based technologies and genomic research in terms of their problematic use as “realities”. Second, we will clarify the definitions and terms of current debates in post-genomic research. Building on these two steps, we will develop an ethical framework of epistemic justice, i.e. fair treatment of the public in the knowledge-related fields of CRISPR/Cas-based technologies and genomic therapies.

P304

AI-Driven Vector Design: Advancements and Challenges in Improving Artificial Intelligence (AI)

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In the past decade, the field of biology has witnessed a remarkable transformation, largely driven by the rapid augmentation of artificial intelligence (AI) technologies. AI has revolutionized various aspects of biological research, enabling scientists to process and analyze vast amounts of complex data with unprecedented speed and accuracy. Vector design plays a critical role in delivering therapeutic genes to target cells but still need optimization for an efficient and more specific delivery. AI algorithms have enabled researchers to optimize vector design by predicting various parameters, such as viral tropism, transduction efficiency, capsid structural viability. Furthermore, data-driven methods can rapidly analyze vast libraries of viral vectors, identify correlation between molecular sequence and phenotypes, and generate novel designs with improved characteristics. The integration of AI into vector has the potential to revolutionize cell and gene therapies by enhancing precision, efficacy, and safety, ultimately resulting in more effective treatments for a wide range of diseases.

However, amidst the tremendous advancements that AI has facilitated, a critical challenge remaining is the control and regulation of AI itself. As AI systems become increasingly sophisticated and autonomous, there is a growing concern about the confidence in their predictions. At WhiteLab Genomics, we consider that controlling AI predictions is essential for integrating AI algorithms into biological research.

Our workflows can be applied to any vector in the field of cell and gene therapy. In this context, we will focus on Adeno-associated viruses (AAV).

- *(i)* A combined approach that couple structural biology with machine learning (ML) algorithms allows us to regain rationality in ML predictions, propelling us beyond the boundaries of traditional interpretation and into a realm of deeper understanding. Notably, we have been able to use our protocol to identify the positions and characteristics of the amino acids that play a predominant role in capsid viability, both in the context of sequence insertions and mutations within the well-known VR-VIII region of AAV vectors and beyond.

- (*ii)* In our classical ML approaches, the use of consensus-like models allows us to control our predictions by leveraging hundreds of models that work together. Our objective is not solely to enhance the predictions, but primarily to pinpoint instances where the certainty of our algorithms diminishes. Consequently, every prediction generated is coupled with a confidence score, which is automatically computed by the algorithms themselves.

The application of these two workflows together demonstrates very promising results, harnessing the remarkable capability to accurately pinpoint AAV variants with unstable predictions, resulting in an impressive 98% performances (with comparable sensitivity and specificity), combined with the identification of specific characteristics that can be used to generate a new vector. At WhiteLab Genomics, we believe that our technology, still under development, will bring a new level of rationality and understanding to predictions.

P305

A double suicide gene therapy using an oncospreading retroviral vector system

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In this study, we used a gibbon ape leukemia virus (GaLV) envelope-pseudotyped semi-replication competent retrovirus vector system (spRRVs) which is composed of two transcomplementing replication-defective retroviral vectors termed MuLV-Gag-Pol and GaLV-Env that encode therapeutic suicide genes. After verifying the antitumor effect of the spRRVs mediated by the double suicide genes on Glioblastoma multiforme (GBM), we analyzed survival rates and tumor burden of six experimental groups (n=8 for each group) established according to the viral dose and prodrug type treated in the rat glioma model. The degree of eradication of tumors and survival curves were used to compare and analyze the correlations for each group or individual. Glioma models were generated by injection of C6 cells into the Wistar Rat brain. After intratumoral injection of spRRV vectors expressing yeast CD and HSV1-TK (in sRRVgp-CD and spRRVe-TK vectors), the prodrugs 5-FC and/or GCV were treated. On the 98th day after tumor establishment, animals were sacrificed and analyzed by histological analysis, and the dose-dependent reduction in tumor size was confirmed. Also, as expected, complete eradication was observed in the test group treated with the highest dose of the therapeutic vectors and both prodrugs. The difference in the treatment effect observed histopathologically was also confirmed through the survival curve data. Finally, the therapeutic effect according to the viral dose and prodrug combination therapy was compared by statistically analyzing the survival rates of the control and treatment groups.

P306

What is the current perception of cell & gene therapy (CGT) among neurologists and revision physicians in Slovakia and Czechia?

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Gene therapies represent 55 % of projects in development, it is only a question of time that they are commonly used. Despite there being more than 1000 patients In Central Europe treated with CGT, based on published and presented data, there is a limited number of reports/publications available to map CGT implementation.
There have been 20 approved CGT in the EU. According to the press releases, some companies did not find a way to successfully introduce these products in the EU and have withdrawn their marketing authorizations, therefore only 12 CGT remain available for use. The number of small companies entering the field with often only one CGT is increasing significantly (currently 47 %) and these new start-ups lack a history of promoting their products. While big companies have established their presence in all countries and launch activities to create awareness of products, including CGT, start-ups are often not present in most countries and realize limited launch activities to introduce new treatments. As a consequence, not all specialists are exposed to recent additions to a variety of available treatments and are not aware of new cell & gene therapies. This situation suggests a gap in knowledge of the current updated CGT availability.

We have collected answers from 34 neurologists and 24 revision doctors and pharmacists from health insurance companies (including prescribers of CGT and decision-makers reviewing CGT submissions) from Slovakia and Czechia about their perception of cell & gene therapy, its effectiveness, and their concerns about using CGT. The questionnaire also mapped their knowledge of available cell & gene therapies in the EU.

For neurologists, the main concern about treating the patient by CGT was related to the patient's individual assessment and shorter experience with CGT. They expressed the need to address topics like prolonging the patient's life without improving it or that it was already too late for the patient to fully benefit from CGT. Neurologists were also sensitive to costs related to CGT.

The revision physicians were predictably concerned about costs but also expressed concern about the shorter experience with CGT. Moreover, the revision physicians agreed that preset 5 years as a long-term horizon in pharmacoeconomic analyses are not sufficient in the case of CGT. The revision doctors lacked clearly defined clinical outcome expectations from CGT and thus evaluating whether the expectations were met.

No one questioned the importance and impact of CGT as improved treatment of untreatable diseases. The revision physicians welcomed the opportunity to share the feedback and additionally shared interest to start the discussion to optimize processes. Neurologists were looking for ways of collecting and sharing experience with CGT among experts, who use CGT.

We are moving from anecdotal use to more systematic and increasing use of CGT. Data showed that clinical outcome expectations outbalanced the cost concern. With the use of CGT, the understanding of CGT improves as well as the knowledge of its benefits. There is still the need to reflect current learnings from use and optimize the processes to limit obstacles in standard use.

P307

Targeted mRNA-LNP encoding IL-15 superagonist complex achieves a delicate balance between efficacy and toxicity in cancer therapy

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Interleukin-15 (IL-15) is an important regulatory factor for innate and adaptive immunity and is considered a promising immunotherapeutic agent for cancer treatment. However, its use is limited due to concerns about toxicity and a narrow administration window. Therefore, we propose the development of targeted delivery systems specifically designed to deliver IL-15 superagonist complex to tumor sites rather than the systemic circulation. This approach aims to maximize the anti-tumor activity while minimizing side effects. The use of mRNA lipid nanoparticles (mRNA-LNP), which have gained significant attention due to their success against the COVID-19 pandemic, has also been widely explored for cancer therapy. mRNA-LNPs offer advantages such as low cost, high transfection efficacy, and potential for clinical translation. In this study, we conducted a proof of concept (POC) experiment using lung-targeted lipid nanoparticles (LNP^{Lung}) to efficiently deliver mRNA encoding IL-15 superagonist complex for cancer therapy in a melanoma metastasis mouse model. Initially, we synthesized hundreds of ionizable lipids with new structures through combinational chemistry. These lipids were then formulated into LNPs by mixing with helper lipids (DSPC: cholesterol: DMG-PEG2000). Through a comprehensive library screening in mice to evaluate both efficacy and safety, we identified top candidates for lung-targeted LNPs (LNP^{Lung}). Next, we optimized the coding DNA sequence (CDS) of IL-15 superagonist complex mRNA using various fusion molecules to enhance its affinity and anti-tumor effects. Subsequently, we evaluated the anti-tumor activity by intravenous injection of mRNA-LNP^{Lung} encoding the optimized IL-15 superagonist complex in melanoma metastasis mice. The results demonstrated that mRNA-LNP exhibited a significant anti-tumor effect and was well-tolerated compared to the positive control (Nogapendekin alfa). Further investigation revealed that the anti-tumor effect was mainly mediated through the promotion of proliferation and activation of CD8+ T cells and NK cells. This finding highlights a promising strategy for cytokine-based cancer therapy, achieving a favorable balance between efficacy and safety through the mRNA-LNP platform.

Key words: Interleukin-15; mRNA-LNP; Lung-targeted delivery; Melanoma metastasis; Cytokines therapy

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P308

High-Throughput process development in rAAV drug substance manufacturing

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The necessity to minimize the timeline to develop commercial gene therapy processes has opened up opportunities for implementing high-throughput (HT) technologies in the field. At uniQure, we have leveraged our platform approach and scale-down models developed, particularly the ambr250 system, for critical process steps to enable automated HT experimentation. The implemented HT strategy has resulted in a reduction in the timeline and costs of our programs. Furthermore, the online data monitored by ambr250 provides an opportunity to understand the kinetics of the process at a higher resolution and accuracy. Additionally, the ambr250 system can automatically operate multiple bioreactors simultaneously. Combined with the design of experiment (DoE)-based approach, ambr250 enables an accelerated optimization of program-specific process parameters and the generation of process knowledge. This new approach and knowledge will be translated into a new generation of rAAV production processes that is even faster and more cost-effective.

P310

Regulating the expression of therapeutic transgenes by controlled intake of dietary essential amino acids: the Nutrireg technology platform

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Widespread application of gene therapy will depend on the development of simple and safe methods to regulate the expression of therapeutic genes. To this end, we have developed a technology for regulating gene expression that exploits the GCN2-ATF4 pathway ability to induce gene expression in response to Essential Amino Acid (EAA) shortage. Briefly, after consumption of a diet devoid of one EAA, the blood concentration of the limiting EAA dramatically decreases, triggering the activation of the GCN2 kinase. It results a rapid induction of the ATF4 transcription factor, leading in the upregulation of specific genes containing an Amino Acid Response Element (AARE) in their promoter. We optimized an AARE-driven expression system called Nutrireg to control the expression of a therapeutic gene by consumption of a diet devoid of one EAA. Nutrireg technology has several unique properties: (1) it does not require a pharmacological inducer; (2) a short term EAA deficiency is not toxic, for longer expression, rotation on several diets lacking one different EAA may be performed; (3) it is rapidly reversible to finely tune transgene expression; (4) it is not activated by drugs commonly used in pharmacy; (5) it is

functional in different organs such as liver, pancreas, eye, adipose tissue and brain. Nutrireg is a technological platform that can be used to treat a wide range of pathologies requiring the controlled expression of a therapeutic gene. For example, we used this technology to regulate the expression of the proapoptotic cytokine TRAIL (TNFSF10) for the treatment of Liver Metastasis from Colorectal Cancer in a murine model. The AARE-TRAIL transgene was packaged into an AAV8 viral vector to target the liver. We first demonstrated the efficacy of Nutrireg to regulate the hepatic expression of TRAIL. Most importantly, liver expression of TRAIL led to a very strong reduction in liver metastasis without hepatic toxicity. It is noticeable that TRAIL is not expressed in the liver of mice fed on a control diet indicating that the transgene expression system is not leaky. Overall, our results represent a solid basis for the promising use of Nutrireg to regulate transgene expression in a reversible way in human targeted organs. This technology exhibits the desirable properties for translation from the laboratory to clinical practice for numerous curative therapeutic possibilities.

P311

Global regulatory landscape of cell and gene therapy products – how to ensure successful global submissions

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The modern technologies to manipulate cells and viruses have enabled development of innovative cell and gene therapy products (CGTs, called in the EU advanced therapy medicinal products, ATMPs) for many diseases with high unmet medical need. The number of licensed products is increasing globally, strongest development has been in the areas of rare diseases, oncology and hematological cancers.

As more products have passed the regulatory review successfully, the interest towards cell and gene therapy has increased and brought also more investments to the field. However, without clear legal and regulatory frameworks, the opportunity to get such complex products to the commercial markets is low. In the US and in the EU advanced regulatory frameworks for CGTs/ATMPs have been in place for more than a decade with increasing number of guidance documents available for general aspects of CGTs, but also for particular product types and technologies. Other jurisdictions are also putting in place regulations for CGTs, but efforts to harmonize the requirements e.g. through ICH are still limited. This has brough challenges for the CGT developers, as there are clear differences between the legal frameworks and also in the authorization processes between jurisdictions, which may hamper wider market access. In the US one single authority (FDA) reviews the information and data of new medicines and follows the development from early non-clinical studies to clinical studies, authorization and up to the post-marketing surveillance. In the EU, the centralized authorization system is built on the expert teams and different authorities of 27 member states, which have different expertise and experience when it comes to review and authorization of CGTs. US FDA has built a Master File system that can be used to share information on starting and raw materials directly to authorities, without releasing confidential information to the customers. In the EU such system is not available, nor are the US DMFs as such acknowledged. This may come as a surprise to CGT developers, who are using e.g. viral vectors or complete media for their manufacturing, ready made by external vendors. Another example of critical differences between US and EU is the regulation of genetically modified organisms, which in the EU is much stricter than in the US and the oversight in many EU members is outside of the medicines authorities. This adds complexity to the authorization process of gene therapy products and requires more extensive risk

assessment than in the US. These are just few examples of the differences one should be aware of when planning for global submissions. This presentation describes the regulatory status on main global markets for CGTs and provides information on specific issues and challenges concerning the regulatory processes and requirements between the US and the EU. The first attempts for global harmonization / convergence of CGT regulations will be also discussed.

P312

Strengthening your viral risk mitigation strategy: impact of ICH Q5A revision

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All biologics require extensive quality control testing and prevention measures throughout their manufacturing process to ensure freedom from adventitious contaminants. The ICH Q5A guidance document, which outlines global regulatory expectations for mitigating the risk of viral contamination, is being revised to reflect recent scientific developments such as new therapeutic modalities, advanced manufacturing and novel detection technologies. As a result, the scope of this document now extends to cell and gene therapy products.

This presentation will consider the impact of the revised guideline on quality control of cell and gene therapy manufacturing, with particular reference to new technologies such as Next Generation Sequencing and PCR-based methods. These technologies offer increased breadth of detection, improved sensitivity and faster timelines. The requirements for implementing such technologies to improve viral safety strategies will be presented, including validation approaches. Opportunities for replacement of *in vivo* and *in vitro* assays with molecular methods will also be discussed. The ICH Q5A revision enables cell and gene therapy developers to realize the benefits of such methods, thus strengthening the ability to detect emerging virus threats as well as furthering ethical and sustainability goals.

P313

Streamlining CMC regulatory acceptance through early involvement of internal CMC & Regulatory team in a Contract Development and Manufacturing Organization

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1: Exothera

Setting a CMC strategy that ensures regulatory acceptance while offering higher agility and swiftness compared to competitors is a challenge for Contract Development and Manufacturing Organizations. The CMC strategy needs to be built on a fit-for-purpose basis, considering the type of medicinal product, the route of administration, the clinical phase of development/ clinical strategy (e.g., pivotal clinical phase), the geographical area, and any product and process technical constraints. These require the proactive identification of CMC gaps and risks at the very beginning

of projects execution to quickly adjust the operational plans during the tech transfer and during process and analytical development phase. The CMC strategy built in partnership with client is fine-tuned during the development, notably based on accumulated product and process data to ultimately be passed to GMP phase. Exothera has built an original and efficient way of working, increasing the CMC regulatory compliance readiness while reducing the time required for GMP batch manufacture preparation and release for clinical use. The project execution model is designed to incorporate the internal CMC & Regulatory team as part of project team from the project set-up up to the final release of clinical batch and submission of regulatory files. This model has already been successfully applied to support and speed up the development phase of adenovirus, AAV and mRNA manufacturing processes. The CMC & Regulatory team works hands-in-hands with Client to de-risk and de-bottleneck regulatory compliance as per project execution lifecycle and as per dedicated services offered.

P314

Innovative therapy in European Parliament's positions: a numerical science-based vocabulary analysis

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The European Parliament has regularly been involved in the framing of innovative therapy, including gene and cell therapy.

Through the evolution of European Union (EU) law, the European Parliament has been more and more involved in the adoption of EU binding law. Indeed, the co-decision procedure (i.e. the joint adoption of legislative acts both by the European Parliament and the Council of the European Union) has become the ordinary legislative procedure from the adoption of the Lisbon Treaty in 2007. But the Parliament has also been involved in other types of procedures such as the consultation or cooperation procedures. Such participation takes the form of documents that are called European Parliament' positions. The latter constitute an interesting corpus to be studied where ones want to know more about the fields of activities of the European Parliament as the European institution representing EU citizens.

Focusing on innovative therapy in a wide meaning, this poster aims at conducting an exhaustive study of the mention of terms related to this topic in European Parliament's positions.

From the official website of EU law (EUR-Lex), we constructed a database comprising all positions of the European Parliament, with available metadata. The database was then searched in order to find every occurrence of a selected vocabulary on innovative therapy, including general terms such as "biomedical innovation" or more specific terms such as "gene therapy", "cell therapy" or "advanced therapy medicinal products". We thus were able to see what terms were used and when they were first mentioned, and in what kind of texts they were most present. In our corpus of texts including our selected terms, another point of interest was the way this vocabulary was interconnected, allowing us to group words according to their co-occurrence in documents. From a legislative mapping perspective, we were also able to see which legal basis were more often used, as well as which documents were citing each other. This work is presented under the form of a network graph comprising all documents and their interrelations.

We finally developed thanks to Natural Language Processing methods a model of all documents containing our selected terms in order to find by means of textual similarity metrics other relevant documents for which these terms could have eluded. This complementary corpus could later be used for further research on innovative therapy in European Parliament's positions, beyond our initially selected vocabulary.

This work has been supported by ANR-funded I-BioLex project (ANR-20-CE26-0007-01).

P315

Initiating an Advanced Therapy Medicinal Product Clinical Trial in the United States Versus in the EU Under the Clinical Trials Regulation (535/2014)

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1: Forge Biologics, Inc.

In 2022, 18% of ATMP clinical trials were ongoing in the EU as compared to 43% in the US.[1] While the US investigational new drug (IND) submission process has remained relatively steady over the last several years, as of January 31st, 2023, all new EU clinical trial applications (CTAs) must be submitted under the EU Clinical Trial Regulation (CTR) (535/2014). The CTR presents potential strategic hurdles for ATMP developers if not considered early in submission planning, which may impact the EU's competitiveness for clinical development with ATMPs. Forge Biologics, Inc. (Forge) will be presenting a comparison of experiences with supporting programs and initiating clinical trials in the US versus in the EU. Specific to the applications in each region, Forge will review procedural and timeline considerations, differences in submission content, and opportunities for cross-referencing quality information. Additionally, Forge will review various regional requirements such as EU Qualified Person interactions, genetically modified organism applications, and in vitro diagnostics requirements in the EU versus those in the US. Forge continues to progress its own therapeutics programs in the US and the EU and will continue to use this experience to support our clients in the navigation of the global regulatory environments.

[1] https://alliancerm.org/sector-snapshot/

P316

The Impact of the In Vitro Diagnostic Medical Devices Regulation (EU) 2017/746 on Advanced Therapy Medicinal Product Developers

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1: Forge Biologics, Inc.

The EU In Vitro Diagnostic Medical Devices Regulation (IVDR) (2017/746) was developed to specify the safety, integrity, and quality requirements for any in vitro diagnostic (IVD) manufactured or used in the EU. The regulation carries few exemptions, including none for

early-phase clinical studies, orphan products, or advanced therapy medicinal products (ATMPs). It came into effect on 25 May 2017 with a 5-year transition period. With the end of the IVDR transition period on 26 May 2022 and the mandatory submission of initial clinical studies under the EU Clinical Trial Regulation (CTR) (536/2014) after 31 January 2023, the IVDR poses major potential hurdles to the development of many ATMPs in the EU.

For ATMPs, anti-drug antibody (ADA) assays are commonly used to determine patient eligibility for clinical trials by characterizing patient immunogenicity to a viral vector capsid and/or the expressed transgene. These assays are considered in vitro diagnostics (IVDs) or companion diagnostics (CDx) and under the IVDR are subject to the full scope of the regulation. This means that the submission and acceptance of performance evaluation plans are needed to initiate participant enrollment in the clinical trial in addition to the approval of the clinical trial application (CTA). In the case of ADA assays for orphan products, there are limited published data or commercial assays to justify an alternative to the development of a CDx, and the ability to assess clinical performance in the patient population is challenged due to limited access to low prevalence patient populations.

Forge will be presenting some of the key hurdles that ATMP developers face under the IVDR and is advocating for additional guidance on the development of companion diagnostics associated with orphan drugs or drugs targeting severe diseases with unmet medical needs (e.g., 'PRIME' products). The IVDR adds a layer of complexity to the conduct of ATMP clinical trials in the EU and necessitates that sponsors give it ample consideration early in the development of their clinical trial strategy.

P317

The challenges of Advanced Therapy Medicinal Products manufacturing in the European Union: Strengths and limits of current regulatory tools

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Advanced Therapy Medicinal Products (ATMPs) is a European classification of medicinal products based on genes, cells and tissues specifically regulated in the European Union (EU) from 2007.

Any company wishing to manufacture ATMPs in the EU must hold a manufacturing authorisation issued by the national competent authority of the Member State where they carry out these activities.

Ensuring the quality of medicines is a criterion to obtain ATMPs' marketing authorisation issued by the European Commission under the "centralised procedure" after a single application to the European Medicines Agency. All ATMPs marketed in the EU must be produced in accordance with EU quality standards to ensure the quality, safety and efficacy of medicines for the patients: Good Manufacturing Practice (GMP) principles and guidelines dedicated to ATMPs enforceable since 2017, and relevant parts of the European Pharmacopeia provide the main regulatory standards to comply with.

The manufacture, meeting the GMP requirements from the raw materials to the final product, is particularly challenging for ATMPs regarding their level of complexity and their specific

characteristics, especially the use of substances of human origin as starting materials, and the reproducibility when using live biological samples. The ATMPs' manufacturer has to maintain costly technical specifications capable of guaranteeing the reproducibility of the medicine's composition in accordance with GMP guidelines and the specific requirements of the ATMP marketing authorisation.

Regulatory requirements are often seen as obstacles to the development of ATMPs. Nevertheless, they mainly provide a set of solutions to overpass the technical obstacles of ATMPs' manufacturing in order to ensure their quality for the safety of patients. It appears clearly within the various supporting regulatory tools currently available at the European level, which include both guidance documents and procedures for interactions with regulators.

This poster will provide an overview of the challenges of ATMPs' manufacturing and link them to the relevant supporting regulatory tools highlighting both their strengths and their limits in the context of ATMPs development for patients.

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P318

Mapping regulators' early interactions procedures to support innovation

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In an effort to help stakeholders to overcome the legal and scientific challenges raised during the development of a medicinal product, procedures, such as scientific advice, have been established in the European Union to enable early contact with regulators, especially in the field of gene and cell therapy. These procedures help to support innovation. They are various: some are available for all medicines and others target innovative medicines or Advanced Therapy Medicinal Products (ATMPs); some are legislative or regulatory schemes, others put into place services; some benefit to all types of medicines' developer, others target a specific type of developer. These procedures for early interactions between stakeholders and regulators are available to facilitate communication between institutions and medicine developers all along the medicine's lifecycle, but the main focus is on the pre-marketing authorisation phase. The exchanges occurring between regulators and the seeker of interaction can impact the chances an ATMP has to enter on the market. Thus, the aim is to foster their development by helping developers to navigate the regulatory pathways adequately for ATMPs to meet quality, safety, and efficacy requirements according to robust evidence to obtain marketing authorisation and access to patients. Knowing all the procedures, who can ask for them, which procedure to use, and with which regulator can be a challenge. Moreover, the extent of the possible support is often overlooked.

This poster describes and discusses the multiple existing options available to ATMPs' developers to overcome challenges during the pre-marketing authorisation phase through early interactions with regulators, here focusing on the European Medicines Agency and National agencies. Communicating on the early procedures available at national and European levels will help to

identify potential gaps and raise developers' awareness of the different procedures available to support the marketing authorisation of ATMPs.

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P319

Mapping the ATMPs guidance landscape

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Relatively new as an autonomous legal category within the European Union (EU) context with the adoption of the Regulation N° 1394/2007 of 13 November 2007 on advanced therapy medicinal products, gene therapy, cell therapy, and tissue engineering medicinal products are also subject of a quite significant amount of soft law production. Indeed, the recent and rapid evolution in these areas and the "youth" of their regulation raise many questions regarding these special rules, their interaction with already existing and more general ones, the requirements for the development of such medicines, their marketing authorisation, their classification, etc. To answer those questions and to dispel as many doubts as possible, guidance documents have been issued by several organisations within the EU context, foremost among them the European Medicines Agency, but also the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) as well as the European Council Directorate for the Quality of Medicines & Healthcare (EDQM) through the publication of the European Pharmacopeia. This soft law that contributed to regulating ATMPs even before the adoption of specific regulation consists in a disparate collection of documents in terms of types (guidelines/notes for guidance, reflection papers, concept papers, points to consider, position statements, questions and answers, etc.), in terms of topics (scientific guidelines, procedural advices, guidance on clinical trials, on production, manufacture, etc.) and in terms of authors as already mentioned. The result is a sea of texts in which it may be laborious to navigate, especially with ATMPs being biological medicinal products and hence *a priori*within the scope of guidance for general and biological medicinal products unless specific guidance exists. This is why our contribution, in the form of a poster, seeks to map a landscape of all relevant guidance available in this field to improve access to it and to facilitate its understanding for developers, marketing authorisation applicants and/or holders, academia, health professionals, etc. First by listing and classifying all institutional authors of these guidance documents identified through internet searches, giving the occasion to assess and to graphically visualise the distribution of each one's contribution in the overall production of such texts, as well as to determine their territorial scope. Second by determining the interactions and overlaps between guidance papers applicable to ATMPs and those applicable to other general or specific medicinal products categories such as biological or biotechnological medicinal products with

which ATMPs share the most guidance in common, as well as immunological or blood/plasma medicines and by representing them in the form of easy-to-read diagrams.

P320

An exploratory analysis on the involvement of European organisations in the field of biomedical innovation

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Many actors influence the framing of EU legislation and regulation applicable to gene and cell therapy. Nevertheless, one can wonder how much their activity is focusing on gene and cell therapy or whether it is part of a wider set of activities taking place in the more general theme of biomedical innovation. From an identification of the actors involved in the public consultation process of adoption of Regulations on Advanced Therapy Medicinal Products, on substances of human origin, and of guidance applicable to ATMPs, a few of them have been selected in order to conduct more specific analysis. Our selection process for this pilot analysis included the following criteria: European level organisation, degree of the involvement in the above-mentioned consultation processes, combination between organisations the names of which imply either a very specific or a general remit. Another choice has been to include European organisations particularly relevant for pharmacists, as professionals involved all along the medicinal products' life cycle. It resulted in a set of 13 European organisations: 5 business associations (Medtech Europe, European Federation of Pharmaceutical Industries and Associations, European Biopharmaceutical Enterprises, European Associations for Bioindustries, European Confederation of Pharmaceutical Entrepreneurs), 2 professional organisations (European Industrial Pharmacists Group, European Association of Hospital Pharmacists), 4 learned societies (European Society of Gene and Cell Therapy, European Society for Blood and Bone Marrow Transplantation, European Eye Bank Association, European Association of Tissue Banks, European Association of Hospital Pharmacists), 1 research institution taking the form of European project (European Network for the Advancement of Clinical Gene Transfer & Therapy), 1 European network based on national medicines agencies (EU innovation network). Then, in depth research has been conducted on the website of each organisation in order to identify their activity within and beyond gene and cell therapy regarding biomedical innovation in general. On the one hand, this poster highlights the main results concerning their organization: especially structure, governance, potential change of name or merger over time, as well as specific or general working committees. On the second hand, their fields of activity are described following an analysis based on a set of keywords. Whereas some keywords are heavily used by all organisations (gene therapy, cell therapy for example), others are more specific (orphan medicine) and others very rare (organoid).

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Comparing actors participating in the adoption process of SoHo and ATMP regulations at the European level

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Genes, cells, and tissues based medicinal products are regulated as Advanced Therapy Medicinal Products (ATMPs) within the European Union (EU). Nevertheless, the very first steps of development of ATMPs, i.e. donation, procurement and testing of human cells and tissues, are governed by the tissues and cells Directive. The latter is being revised and the European Commission has adopted a proposal for a new regulation covering Substances of Human Origin (SoHO) more widely. Both the regulation on ATMPs and the current proposal for a regulation on SoHO, as EU legislative acts, have involved a public consultation procedure to gather comments from stakeholders and interested parties. How similar or different are the actors that influence the framing of EU legislation applicable to gene and cell therapy?

Our poster aims at mapping these actors for both ATMPs and SoHO, and their potential changes over time, to identify possible differences and/or convergences as to who exactly has been involved in ATMPs and SoHO regulations.

The identification of answers to the relevant public consultation procedures has led to the establishment of a database with 530 contributions corresponding to both ATMPs and SoHO regulations' documents. The participating organisations have been classified by type (Business, Academia, National bodies, etc.) and country according to the documents they commented on.

Findings concern firstly the main differences between SoHO and ATMPs regarding participating organisations. Secondly, some organisations also revealed themselves as particularly active in both areas of ATMPs and SoHO, which highlights their interest for the entire development cycle of ATMPs and its related legislation.

Contrasting the repartition of stakeholders interested in ATMPs and SohO may indeed explain some of the differences in the way the regulation is being constructed and its level of detail: the more a type of organisations is involved, the more it has a chance to impact the regulation's framing according to its own specific interest.

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Stable and inducible AAV producer cell lines to support large scale AAV manufacturing

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Innovative strategies are required to develop large scale manufacturing solutions and provide commercially viable AAV products to large clinical populations. Current AAV manufacturing platforms utilize suspension cultures of mammalian- or insect-cell origin, with extensive engineering efforts underway to address their limitations, including high plasmid cost and scalability in mammalian platforms and baculovirus instability in insect cell-baculovirus expression vector (IC-BEVs) platforms. To simplify and improve the efficiency of the insect-cell manufacturing system, we have developed the first stable and inducible AAV producer cell line system using the OneBac IC-BEVs platform. OneBac producer cell lines are engineered with inducible promoters, enhancer motifs, and proprietary Kozak sequences to enhance capsid stoichiometry, overcoming limitations of reduced potency of AAV vectors derived from the IC-BEVs system. Stable integration of these elements into the Sf9 genome enables robust, high titer AAV production following infection using a recombinant baculovirus devoid of AAV elements thereby mitigating BEV genome stability issues during scale-up. The system is adaptable to multiple AAV capsid serotypes and effectively packages self-complimentary and single-stranded AAV genomes. Image verified clonal producer cell populations can be rapidly screened to identify rhabdovirus-free candidates capable of exceeding E5 AAV gc/cell or E14 AAV gc/L. Process optimization has enabled the potential to screen hundreds of clonal candidates for any given AAV product with development times currently around 3 months, from stable DNA integration, single cell isolation, clone screening, and scale up to cell banking. The manufacturing performance of OneBac producer cell lines is reproducible and consistent, studied in the context of multi-passage productivity studies and the derivation of subclonal populations, supporting stability and linear scalability of the system. Next generation sequencing of AAV products derived from the OneBac platform indicates minimal encapsidation of process-related impurities such as host-cell and baculovirus DNA. Importantly, OneBac-derived AAVs are as potent and effective as their mammalian-derived counterparts in multiple *in vivo* disease models. In conclusion, this novel producer cell line system is a highly promising solution for low cost, robust and highly reproducible large scale AAV manufacturing.

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Scale-up and GMP manufacture of AAV in suspension 293T/17-based cells

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Production of AAV for early-phase clinical use requires manufacture utilizing hundreds to thousands of liters of upstream cell culture. Production facilities for early-phase trials have often relied upon adherent cell-based processes, though suspension cells offer greater flexibility and far superior scalability. We developed a transfection-based process using 293T/17 cells adapted in-house to suspension culture. Cells were cultured with LV-MAX Production Medium in 5 L glass or 200 L disposable Sartorius bioreactors and transfected using TransIT-VirusGen. Two days post-transfection, cell lysis was performed using high-pressure homogenization and the combined media and lysate were clarified via depth filtration. Filtrate was loaded directly onto a POROS AAVX affinity column, after which additional impurities were removed via hydrophobic interaction on a Sartobind Phenyl membrane. After buffer exchange, anion exchange chromatography on a BIA CIM QA monolith was utilized to enrich genome-containing AAV particles. For GMP manufacture of Phase I/II material, four 200 L bioreactor runs were purified through anion exchange chromatography, buffer exchanged to PBS, formulated in 0.25% recombinant human albumin, and frozen. These four runs were subsequently combined into a pooled drug substance, diluted to a target concentration of $5x10^{12}$ vg/mL, and filled into 10 mL glass vials.

Average titers across four 5 L runs were $1.74x10^{11}$ vg/mL and $8.20x10^{11}$ capsids/mL, while average titers across five 200 L runs were $1.65x10^{11}$ vg/mL and $8.39x10^{11}$ capsids/mL. Average genome recovery from harvest to final product was approximately 15% across the four GMP runs, with a final production yield of $1.96x10^{16}$ total viral genomes. Other results, including cell growth and impurities, were consistent across 5 L and 200 L scales and will be discussed along with several challenges related to scale-up and manufacture.

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Using mechanistic models to design a platform process for the separation of full and empty AAV capsids

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During the upstream production of adeno-associated viruses (AAVs), separate cellular processes are used to produce the viral capsid and the therapeutic transgene, and package the transgene within the capsid. This leads to the production of empty capsids which must be removed during the downstream process as they may add to immune stimulation while not providing vector genome delivery.

The separation of empty capsid presents a challenge due to the similarity in properties between the empty and full capsids. The proportion of empty capsids can vary widely and be as high as 90% depending on the maturity of the upstream process which can further increase the challenge in achieving this separation. Most attempts have focused on using anion exchange (AEX) chromatography to exploit the difference in charge between empty and filled capsids to achieve this separation.

However, there is a lot of diversity in the published approaches with some groups using different matrix types (resins, membranes, and monoliths), process conditions, and additives. We have previously demonstrated that weak partitioning can be used to maximise the enrichment of full capsids which further increases the available options for this separation.

Establishing a platform process typically involves screening a number of options while using heuristics to narrow the design space and reduce the experimental burden. This typically leads to process options being compared at sub-optimal conditions and there is a risk that the optimal platform may not be identified.

We demonstrate that mechanistic models can be used to identify an optimal platform process for the enrichment of AAV2 full capsids. In this study, we developed mechanistic models for the separation of full and empty AAV2 capsids for three AEX matrices to that had previously shown promise during experimental evaluation.

These models were then used to examine a range of process conditions (salt concentrations, load ratios) and the modes of operation (bind and elute, flowthrough, weak partitioning) and identify the optimum conditions for each matrix. Finally, the identified optimum conditions were verified experimentally.

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AAVone: an all-in-one plasmid system for efficient AAV production

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Recombinant adeno-associated viral (AAV) vector production is seen as complex, with the production scale-up regarded as a major challenge technically, and a large barrier for commercialization. Currently, transient transfection of plasmid DNA into mammalian cells (such as HEK293) is the strategy most used in manufacturing of clinical grade AAV vectors. Typically, three - plasmids are used: one carrying the regulatory (Rep) and structural capsid (Cap) genes, one carrying the ITRs and transgene, and one carrying genes that provide helper adenoviral function. The transfection approach is rapid and versatile and has been used to produce different serotypes of AAV. However, producing GMP grade of three plasmid with high consistency, high purity and free of process-related impurities and variants remains a challenge in AAV production. Here, we developed a novel AAV production system, named AAVone, in which only one plasmid is needed. It increases AAV yield, improves production consistency and reduces the cost and labor. Firstly, we created a mini-pHelper with size of 8.4 kb by deleting the introns of E2 and E4 expression cassettes. Secondly, we integrated eighter AAV helper genes (Rep and Cap) or transgene genome into the mini-pHelper and created two versions of dual-plasmid systems (Dual-V1 and Dual-V2). Finally, we incorporated all the necessary elements into one plasmid. Thus, in the AAVone system, Ad helper genes (E2A, E4orf6 and VA RNA), AAV helper genes (Rep and Cap), and AAV vector genome are assembled into one plasmid and AAV vectors can be simply generated by transfection one plasmid into host cells. AAVone system achieved a productivity of 0.5~1.2×10^{15} viral genome/L(VG/L) for AAV2 and over 2×10^{15} VG/L for AAV9, which is 2~4 fold higher than triple-plasmid system. Moreover, AAVone has low batch-to-batches variations, as it only needs one plasmid successfully transfected into a cell, without the need to co-transfection of the other two. AAV vectors generated by AAVone and traditional triple-plasmid system have the same capsid and genomic components and infectivity, comparable levels of Rep, Cap, rcAAV genomic contaminations, but less plasmid backbone genomes. Moreover, AAVone system uses less total amount of plasmid DNA, and avoids the ratio optimization steps comparing with the triple-plasmid system. Taken together, AAVone is a simple, low cost, but high consistency and high efficiency AAV production system especially for GMP grade AAV production.

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Scalable manufacturing of cell therapy products from human stem cells

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Stem cells and stem cell-derived products (extracellular vesicles, EV) provide an outstanding therapeutic perspective for multiple diseases. To ensure translation into viable products, established lab-scale cell proliferation procedures have to be evolved into scalable manufacturing

processes. Phoenestra currently develops technology platforms to meet this rising demand of the gene and cell therapy field.

We have developed a cultivation process for induced Pluripotent Stem Cells (iPSCs) that applies shake flask cultivation as intermediate scale to seed a bioreactor which is then used in perfusion mode to reach cell densities in the million per milliliter range.

Multipotent Mesenchymal Stromal Cells (MSCs) are promising for therapeutic applications in various diseases and tissue repair (immunomodulatory and regenerative properties), but scalable cultivation of these adherent and sensitive cells is challenging. We have developed a stable process that uses cell carriers inside an agitated packed bed system (experimental model) in DASbox bioreactors (250 mL) and have successfully produced >10^{12} EV particles per month from stable MSC lines.

To better inform downstream process design we have started to systematically investigate surface interactions, storage conditions and freeze-thaw cycles on MSC-derived EVs and identified critical and non-critical factors for EV handling. Methods such as flow cytometry, functional bioassays, and nanoparticle analysis, etc. enable detailed characterization (quantification and sizing, marker profiling etc.), to detect relevant biological activities and to explore modes of action. Overall we present end-to-end approaches for scalable EV manufacturing from different cell sources.

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Successful development and scale-up of an AAV production suspension platform

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Recombinant adeno-associated virus (rAAV) vector is the most widely used viral vector for *in vivo* gene therapy thanks to its safety profile, efficient transduction to various tissues and long-term expression after a single injection. Transient transfection of suspension HEK293 cells is one of the most used method for preclinical- and clinical-grade rAAV manufacturing, due to its versatility for different serotypes and its scalability from research to commercial scale. However, the manufacturing of rAAV viral vectors remains challenging to meet safety and efficacy requirements, as well as clinical and market demands, including the necessity of an affordable cost.

Our work aimed to develop a powerful rAAV production platform based on transient transfection of HEK293 cells in suspension, which combined an efficient upstream process able of delivering large amounts of rAAV and a flexible downstream process to meet specific product requirements (concentration and purity).

After only a few months of small-scale development through a holistic approach, an end-to-end process was defined. This development covered the definition of the best raw and starting materials such as cell line, transfection reagent and media, innovative technologies and appropriate consumables, and critical process parameters.

This so called “TaRGeT’s AAV platform” showed high and consistent productivity up to 5x10^{11} vg (vector genome)/mL in bulk harvest. The downstream process performed through several steps

(clarification, optional concentration, affinity chromatography, optional ion exchange chromatography, concentration, and final sterile filtration) resulted in a concentrated product (> $5x10^{13}$ vg/mL) with an overall high yield, as well as with the targeted quality attributes. Finally, this process has been successfully scaled-up from shake flask to 2L then 50L stirred-tank bioreactors.

The final yield obtained from a 50L batch reached a total of $1.25x10^{16}$ vg, which is in line with the current clinical amounts needed of rAAV-based viral vectors (with a dosing ranging from 10^{10} to 10^{16} vg per patient, depending on the therapeutic area). This successful development will support our internal portfolio, but also external partnerships in order to accelerate patient access to new gene therapies.

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Successful scale-up validation of a small-molecule compound for increased AAV yields

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While recombinant adenovirus and retroviral vectors dominated the early years of gene therapy development, recombinant adeno-associated virus (rAAV) is most widely used for *in vivo* gene therapy today. However, one of the major challenges associated with AAV gene therapy is the cost-efficient production of high-quality vectors meeting safety and efficacy requirements in a rapidly evolving regulatory environment and for more common diseases with higher hurdles regarding risk/benefit profiles. To address this issue, we applied a high throughput small molecule screening strategy in suspension HEK293 cells using the ATLAS (Arrayed Targeted Library for AAV Screening) platform to identify compounds that demonstrate enhanced rAAV production. Targets identified included transmembrane proteins, DNA repair proteins, cell-cycle regulators, and epigenetic modulators. After a series of studies in small and large-scale shake flasks, we identified a novel compound that increases rAAV production in a robust and dose-dependent manner.

We present here validation data generated in a controlled bioreactor predictive of our large-scale manufacturing platform supporting the scalability of this novel compound with respect to productivity and without compromising vector quality. Using the Ambr® 15 bioreactor system, we confirmed the robust and dose-dependent increase of rAAV yields previously demonstrated in the shake flask format. As the cells, plasmids, transfection parameters, media and the overall design of the upstream process defines several critical vector quality attributes which cannot be significantly improved by down-stream purification, we additionally analysed the impact of small molecule addition on selected quality parameters. Our data show that vector quality is not compromised, indicating analytical comparability of vectors produced with our next generation platform.

In summary, we validated the applicability and scalability of the novel small molecule compound for high quality, high yield rAAV in our manufacturing platform. Validation of additional hits that have shown to drive AAV yields in shake flasks is currently ongoing in our small-scale bioreactor platform.

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How uniQure's modular platform approach drives continuous innovation in AAV gene therapy development and manufacturing

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uniQure's BEVS platform for AAV manufacturing is approved for commercial supply. Having an approved platform is a great asset. The key benefit is that data can be shared between products using the same platform. For example, studies on viral clearance, leachables/extractables or process characterization can be leveraged across products or may require a small bridging study to justify product-specific changes. This reduces time to clinic and reduces cost and resources throughout CMC development. The risk is that one might avoid introducing improvements into the platform because that may result in the loss of this benefit, effectively locking the platform with outdated technology and making improvement difficult. To ensure that the platform keeps evolving, uniQure has developed a modular approach towards its manufacturing platform. The platform is divided in 5 modules: Starting Materials, Upstream-, Downstream- and DP-process and Analytics, each consisting of several unit operations. Generations of the platform are defined at the module level. Impact of changes on are assessed at an early stage to allow studies off the critical path. The GT field is rapidly maturing. As a result, there is more demand for advanced technologies. This requires a flexible approach and rapid introduction across pipeline products and subsequent stages of development within existing programs. This presentation provides an overview of uniQure's platform approach for continuous innovation and gives examples of how new technologies are introduced and implemented in pipeline products.

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Current approaches and considerations for viral clearance in cell and gene therapy (CGT) manufacturing processes

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The assurance of viral safety in cell and gene therapy (CGT) products poses a unique challenge as the viral vector is a key component of both *in vivo* and *ex vivo* gene therapies, creating unique challenges to clear or inactivate potentially present adventitious viral agents from the manufacturing process, when the therapeutic product itself is a virus. Although viral clearance strategies for general biological drug manufacturing and vaccine production will be applicable to these products, there will be unique challenges and considerations for CGT modalities.

BioPhorum Cell and Gene Therapy (CGT) is an industry-wide consortium that supports the quest for better and faster development of cell and gene therapies. The BioPhorum CGT validation workstream published a white paper in June 2023 that details in a single location the major relevant existing guidance and advice on viral clearance for viral vectors with an emphasis on

adeno-associated viral (AAV) vectors. This works highlights the unique considerations for CGT, provides potential options for resolving these challenges, and proposes a 'gold standard' approach for viral clearance for the manufacture of cell and gene therapies.

Key thematics and messages from the paper will be presented here, including the regulatory landscape, phase appropriate considerations, viral clearance and inactivation mechanisms, suggested strategies for viral clearance validation, and modes of viral clearance.

We believe that these approaches will be a valuable resource for the CGT sector in order to continue to ensure that CGT remain safe for patients, through sharing the experience and expertise of the BioPhorum CGT member companies, and leveraging well-established approaches for viral clearance from other applicable therapeutic modalities.

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Tech transfer and scale-up: A case study of Exothera's collaboration with Cytiva to yield two new facilities and AAV proof of concept

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To tackle today's AAV manufacturing shortage and large-scale capacity, Exothera built two new facilities and reached GMP certification in less than two years thanks to critical collaborators. Cytiva was chosen as a supplier for both upstream and downstream processes thanks to its broad range of systems. Cytiva's initial support was key in Exothera's production-line readiness, with continuous support in URS answer and system customization, manifold workshops for full production set creation, and final logistics handling and validation. LogicBio Therapeutics then worked with Exothera for the scale-up of an AAV8 vector manufacturing process using Polyplus's FectoVIR® for transient transfection.

For the upstream process, Cytiva and Exothera optimized the AAV8 production process to fit industrial scale. Historically, Exothera produced 6.98 × 10^{11} viral genomes (vg)/mL in an Eppendorf® BioBlu® 50 L bioreactor. To transfer the process to our technology platform, we focused our strategy on tailoring parameters such as power input per volume and superficial gas velocity for a stable oxygen transfer rate. This would also allow for a complete and predictable scale-up throughout our bioreactor range. This new method led to a successful first 200 L batch, with a product titer reaching 6.85 × 10^{11} vg/mL. By applying the same scale-up plan to 2000 L scale (using a 200 L bioreactor as seed train), the product titer reached an outstanding 8.20 × 10^{11} vg/mL, making it the most productive run at manufacturing scale with this process, outlining the success of our tech transfer method and application in this key collaboration.

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Investigating the impact of CD19-CAR inclusion into the lentiviral particle envelope, as an upstream byproduct, on ion exchange chromatography recovery rate

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The manufacturing process of lentiviral (LV) vectors relies on providing vector-component blueprints, for example in the form of plasmids, to the producer cell line. This principle does not apply, however, to a key structural component of the LV particle, the membrane envelope. The envelope composition is dictated instead by the membrane composition of the producer cell line during the budding phase. This is especially relevant for manufacturing LV vectors encoding chimeric antigen receptor (CAR) transgenes, since their translation and subsequent incorporation into the cell membrane of the producer cell can take place. As a consequence, this upstream byproduct can become part of the lentiviral particle envelope. Here, we aim to investigate if the inclusion of CAR proteins into the LV membrane envelope, during the upstream process with a suspension cell line, impacts the ion exchange chromatography recovery rate. In this study, we have used a benchmark CAR, a second generation CD19-CAR, like the one in the prescription drugs Yescarta®/Tecartus®. As a transgene control that is not incorporated into the producer cell line membrane during production, we used GFP. First, we confirmed by western blot analysis that the CD19-CAR LV vectors manufactured with our upstream conditions contain both VSVG glycoprotein and the CD19-CAR. Next, we tested whether the presence of CD19-CAR in the LV vector envelope surface impacts the recovery rate of ion exchange monolithic chromatography. For the CD19-CAR vector, we have observed a 46.9% ± 5.3% mean recovery rate (n=3), while for the GFP vector a 36.8% ± 4.0% recovery mean (n=3). This indicates that CD19-CAR protein does not compromise the recovery over the ion exchange monolith. We also polished the particles using a multimodal chromatography resin and obtained a mean recovery rate of 32,2% ± 1,5% (n=3) and 21,5% ± 1,4% (n=3) for the CAR and GFP vectors, respectively. As with the monolith, the recovery was not affected by the surface CD19-CAR. Further work is planned to test if CD19-CAR inclusion in the LV membrane envelope influences an affinity purification method based on agarose-beads e.g., CaptureSelect™ Lenti VSVG Affinity Matrix as well as subsequent polishing and formulation steps. Also, we plan to investigate the effect of the CD19-CAR byproduct levels on the LV vector transduction efficiency and tropism. This work will provide important insights to optimize the manufacture of LV vectors used for developing CAR-T therapies.

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Rational engineering of CpG-free DNA elements for gene therapy

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Non-methylated cytosine-guanine dinucleotides (CpGs) in foreign DNA sequences can be recognized by the Toll-like receptor 9 (TLR9) leading to acute inflammatory responses. In immunotherapy, CpG oligodeoxynucleotides have been utilized as immune stimulants. In gene therapy, however, it is essential to prevent the induction of an immune response to enable a sustained and safe therapeutic effect. Elimination of CpGs in plasmid DNA expression vectors and AAV vectors has been shown to be beneficial in mouse studies to circumvent immune responses and achieve persistent transgene expression compared to CpG-containing vectors. Furthermore, the impact of CpGs in codon-optimized transgenes on the loss of transgene expression in a hemophilia B clinical trial is currently discussed. Here, we generated a CpG-free version of (i) the liver-specific transthyretin (TTR) promoter, (ii) the U6 RNA polymerase III promoter, and (iii) the single-guide RNA (sgRNA) scaffold, which could potentially improve the immunological outcomes of gene therapy applications. The sequences of our CpG-free TTR and U6 promoter are based on alternative sequences found in different mammalian organisms (NCBI, RefSeq Genome Database, mammalia, taxid: 40674), as we assumed that these base exchanges would not affect functionality. As there are no alternative sequences for the sgRNA scaffold available, we aimed to conserve the secondary RNA structure. The resulting sequence displays nearly identical secondary RNA structure with equivalent levels of free energy. *In vitro* functionality of the CpG-free TTR promoter was confirmed in a luciferase reporter assay and similar levels of expression were obtained. The liver-specificity is maintained, as shown by a comparison of expression in hepatic and nonhepatic cell lines. Furthermore, we validated the *in vivo* functionality of the CpG-free TTR promoter to initiate gene expression in BALB/c wild type mice after luciferase gene transfer to the liver. For the functional testing of the CpG-free U6 promoter and sgRNA scaffold, we used a HEK293 cell reporter system in which GFP is activated after successful adenine base editing. The CpG-free U6 promoter, initiating transcription of the sgRNA, resulted in the same editing efficiency as the CpG-containing U6 promoter, proving that there is no functional difference. In contrast, the CpG-free sgRNA scaffold presented a 49.4 ± 6 % reduction in editing efficiency, suggesting that other factors besides secondary structure determine functionality. In conclusion, we successfully generated CpG-free promoters based on sequence comparisons. Future work will include the implementation of a bioinformatics algorithm to automatize CpG-replacement and facilitate the generation of further CpG-free DNA elements. The expected immunological advantage of our constructs is currently based on the well-known relationship between CpGs and the activation of immune responses and will be studied in future experiments. The CpG-free TTR promoter could provide an advantage in liver-targeted gene augmentation therapies, while the CpG-free U6 promoter and sgRNA scaffold could find application in the field of genome editing.

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Improving the elution step in capture chromatography: a mechanistic approach

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Bulk recovery of adeno-associated viruses (AAV) in the capture step is often performed using affinity chromatography. Whilst this technique offers great capsid adsorption, the final recoveries can be lower than expected (< 80%). This disparity between loaded and recovered product suggests there are mechanisms not fully understood in affinity chromatography. To understand this operation a Design of Experiment (DoE) was performed, using an AAV5-based product, to determine the main factors affecting the elution behaviour. It was found that weak acid solutions and the presence of solvents increase the recovery yield of AAV. It was discovered that at certain

pHs the viral genome (VG) recovery decreased without a corresponding decrease in virus particle (VP) recovery. A genome escape mechanism affecting the recovery of AAV genome copies is suggested, potentially explaining the discrepancies between VG and VP mass balances.

A second DoE was then executed to characterise the process parameters affecting the elution of the AAV5 product. The findings from the previous DoE were confirmed with this second model, proving that solvent use, acid type, and pH were the main factors contributing to a yield increase. It was established that small changes in the pH led to a greater than two-fold increase in the recovery yield. These small changes were confirmed with other therapeutic vectors (AAV2 and AAV8).

This work offers insights into the underlying mechanism of AAV elution from affinity chromatography, and how small changes in the process parameters can greatly increase the bulk yield of a therapeutic product. It also highlights a potentially novel ejection mechanism that can describe unexplained yield losses during the capture step.

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Scalability and bioreactor supplier change for GMP lentiviral vector manufacturing in a suspension cell culture system

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An important challenge in the manufacturing of lentiviral vectors (LVV) is to be able to provide different quantities of high-quality vectors for different needs, in a scalable manner that ensures comparable yield and product quality. To this end, we have recently launched a suspension lentiviral vector manufacturing process platform based on quadruple plasmid transient transfection of HEK293T cells grown in bioreactors in serum-free conditions at 50L and 200L scale, called Lentisure™. This platform benefits from more than 10years of cumulative experience in LVV manufacturing at Yposkesi.

However, the quantity of LVV obtained at 200L bioreactor scale with our process is often in excess of actual CMC needs for new therapeutic approaches, such as for cancer immunotherapies (CAR-T or TCR cells), especially at early stages of development. As such, we have scaled-down our 200L bioreactor scale lentivirus manufacturing process to a 50L bioreactor scale, while also switching to a different bioreactor equipment supplier, in order to adapt to these lower needs of high-quality vectors and at a more competitive cost. The bioreactor supplier change (new culture bioreactor technology) was performed for reasons of operational flexibility.

For the scale down approach, the cell culture conditions were optimized taking into account bioreactor geometries and the different gassing and mixing capacities of the new bioreactor platform. All other process parameters and steps were also reduced/scaled back (from 200L scale to 50L scale) to fit with the harvested volume to be purified (50L).

We demonstrate that the cell culture performance and product quality in both bioreactor systems were similar. Results will be presented for infectious viral titre (IG/mL), particle content (ng p24), protein expression (Flow cytometry) and process related impurities (protein and DNA). The work will demonstrate that the product quality profile of the lentiviral vectors produced by both suspension manufacturing processes at 50L and 200L are comparable, with good correlation for

cell culture process performance, viral titre, and product quality. Finally, the results are also compared to similar data from the Lentisure™ LVV adherent manufacturing platform, which further show that the quality of the LVV produced is not impacted by the process (adherent or suspension) or scale (50L or 200L suspension) used for LVV manufacturing with the Lentisure™ platform.

In conclusion, we have scaled down an LVV suspension manufacturing process from 200L to 50L Bioreactor scale, while changing equipment supplier. Process performance and product quality was comparable between scales and equipment, and also between adherent and suspension manufacturing platforms, which highlights the adaptability of the LentiSure™ platform to adapt to changing needs (lower quantities, same quality) for LVV clinical programmes.

P336

neDNA™ is a robust alternative to plasmid DNA for AAV production

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1: Viralgen Vector Core

The most popular method for production of recombinant adeno-associated virus (AAV) vectors is through transfection of three plasmids, one containing the gene of interest flanked with AAV inverted terminal repeats, one encoding the AAV rep and cap genes, and one encoding the adenovirus helper functions. However, the use of plasmid DNA leads to contamination of the AAV products with sequences of bacterial origin that may cause safety issues. As an alternative, these three sequence elements can be provided by other DNA starting materials. In this study, we evaluated the use of neDNA™, i.e., linear DNA molecules with closed ends that do not contain bacterial sequence elements, compared side-by-side with plasmids, for AAV production though triple-transfection of the Pro10™ cell line. We found that both types of starting materials achieved similar vector titers and quality, both at the 2-liter and at the 50-liter scale. Furthermore, vectors produced with both types of DNA and carrying the same transgene sequence were injected in mice, and vector distribution and transgene expression was followed for four months. Overall, the results show similar transgene distribution and expression level regardless of the starting materials. This confirms that neDNA™ may offer an interesting alternative to plasmids for production of AAV vectors.

P337

Assessing the potential of the ELEVECTA· HEK293 platform for AAV manufacturing with a cost modelling approach

E Cameau [1] M Mühle [1]
1: Cytiva

To address the growing demand for industrial scale adeno-associated vector (AAV) production, we have recently launched an innovative stable, helper virus-free AAV production platform called ELEVECTA. The ELEVECTA stable producer cell lines harbor all relevant components for AAV production stably and inducibly integrated in their genomes, which enables high-titer AAV production upon addition of the inducing agent.

Cell line engineering is a key area of process optimization in order to increase overall productivities, and to ultimately decrease manufacturing costs of these 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 platform has been established.

In this work, we have modeled several different scenarios of process optimization based on the ELEVECTA platform, and we look at how they impact the cost of manufacturing compared to the baseline process. Scenarios include faster growing cell lines, cell lines producing a higher ratio of full capsids vs. empty, among others.

P338

Optimizing Scale-up of AAV Gene Therapy in Upstream Processing

S Gomez [1] R Garus [1]
1: Viralgen Vector Core

Viralgen specializes in the production of Adeno-Associate Virus (AAV) gene therapy vectors using our proprietary suspension, triple transfection platform. This includes a Human Embryonic Kidney (HEK)293-derived suspension cell line, a scalable upstream and robust purification process, coupled with full support for fill and finish, Quality Control testing, and regulatory support from preclinical to commercial requirements. We have recently completed studies to achieve the 2000L scale-up which have been continuously optimized through both process characterization and experimental approaches to better understand key process steps such as mixing dynamics and transfection cocktail maturation kinetics. All knowledge is reintroduced into the process to improve yield and recovery while also maintaining product quality.

P340

neDNA™, a robust and high-quality DNA supply for rAAV manufacturing

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1: TAAV Biomanufacturing Solutions

TAAV-manufactured enzymatic neDNA™[1], is a critical starting material that provides comparable productivity and potency to plasmid-derived recombinant Adeno-Associated Vectors (rAAV)

vectors and increases efficiency compared to plasmid, as less input DNA is required in transfections.

neDNA™ manufacturing process is essentially an *in vitro* dual enzyme process: Phi29 polymerase for rolling circle amplification of the precursor template, and TelN protelomerase for covalent closure of both ends. High throughput sequencing (HTP) analyses on neDNA™ final product have demonstrated that an intended sequence is amplified with high fidelity, including complex molecular structures such as AAV inverted terminal repeats. Moreover, High-Pressure Liquid Chromatography (HPLC) results have shown that neDNA™ final product has a high degree of purity (>95%).

The removal of bacterial sequences is anticipated to improve the safety profile of rAAV products. neDNA™ has virtually no bacterial backbone, and HTP and quantitative PCR analyses of neDNA™ final product have consistently shown <1% Kan^R residual sequences. neDNA™ bears the DNA sequence of interest, TelN recognition sites at both ends, and a stuffer sequence that enables measurement of any residual neDNA™ in final rAAV products. Asklepios BioPharmaceutical has shown *in vivo* that the stuffer is inert and that it does not have any transcriptional activity.

In summary, we believe this study highlights the advantages of using neDNA™, a critical starting material with high-quality and minimal contaminants, that may resolve the challenges in rAAV production, since enzymatic DNA amplification offers rapid production, scalability, and robustness.

[1] Technology for making neDNA™ is licensed from Touchlight IP Ltd.

P341

Identification of universal parameters that play a critical role in rAAV production using a transient two-plasmid packaging system in the adherent HEK293T cell line.

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Adeno-associated virus (AAV) is the vector of choice for gene therapy due to its relatively good safety profile and persistence, which has led to a booming number of clinical trials. To meet the increasing demand, challenges in manufacturing of recombinant AAV (rAAV) vectors such as low yields and low full:empty capsid ratios need to be tackled.

In this work, we studied the effect of critical process parameters that determine the yield and quality of rAAV production. We started from a baseline rAAV production protocol based on the adherent human embryonic kidney (HEK)293T cell line in combination with a two-plasmid transient transfection system in which *rep* and *cap* genes and Ad helper genes are on the same plasmid. As this protocol routinely yields decent yields and full:empty ratios, we considered it a good starting point for identifying the process parameters that lead to superior rAAV production yields. We evaluated the effect of plasmid quantification, confluency at the time of transfection, total plasmid DNA (pDNA) amount, DNA:PEI ratio, and plasmid ratio on vg titer, VP titer and % full for AAV2, AAV5, and AAV9. In addition, we measured the intracellular average copy number

of the different plasmids 16h after transfection in HEK293T cells to allow monitoring of the effect of aforementioned parameters, both on transfection efficiency as on AAV production. In an effort to obtain more reproducible transfection conditions, we found that determining pDNA concentrations using the Invitrogen Qubit HS assay was more reliable than using a Nanodrop spectrophotometer. Most likely, this is due to the tendency of traditional UV absorbance-based methods to overestimate sample concentrations due to the presence of contaminants. We found that there is an optimum in terms of amount of total pDNA and the DNA:PEI ratio at which maximum transfection efficiency is obtained, which correlated well with intracellular pDNA copy numbers and the % full particles. Moreover, an inverse relationship between total vg and % full particles was observed, meaning that parameters that increase total vg concomitantly decrease % full and vice versa. Independent of the serotype, the best results for rAAV production were obtained by decreasing the helper:production plasmid ratio, with a gradual decrease in total vg though concomitant increase in % full.

In summary, we found that UV absorbance-based methods for pDNA quantification do not form a reliable basis for rAAV production process development, and that monitoring of intracellular pDNA copy numbers allow for better insight into the transfection and production process. Moreover, we observed an inverse relationship between total rAAV particle yield and percentage full. The driving force behind this relationship is yet unclear and the subject of follow-up research.

P342

How to improve Lentivirus polishing recovery using Capto Core 700 resin

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1: Cytiva

Lentiviruses are enveloped RNA viruses commonly used for the rapidly growing field of chimeric antigen receptor (CAR) T cell therapy. In this therapy, T lymphocytes are genetically modified by lentiviruses *ex vivo* to express a CAR receptor. Once the lymphocytes are administrated *in vivo* in the patients, they target and destroy cancerous cells. Purification of lentiviral vectors is very challenging due to the low stability of these enveloped viruses, which are sensitive to low pH, high salt, temperature, shear forces, and other factors. In the different bioprocess steps from harvest to final sterile filtration, efforts must be made to minimize conditions that negatively affect a good physical and infectious titer recovery. Lentivirus purification is often carried out using anion exchange capture chromatography but typically with losses in physical and infectious titer recovery. Here, we present optimized capture from clarified feeds using weak anion exchange with Capto™ DEAE resin followed by a Capto™ Core 700 resin polishing evaluation with different buffer pH values and flow rates. We show that using pH 7.0 significantly improved infectious recovery while the physical recovery remained similar. We also show that residence times down to 0.7 minutes can be used without negatively affecting the infectious recovery. The impurity removal for the Capto™ Core resin polishing step was similar with total DNA and protein below the level of quantitation for all conditions.

P343

Scalable ultra-purification of AAVs: a novel standard to boost transduction efficacy and potency for cardiac gene therapy development

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A high transduction efficacy and potency of AAV-based cardiac gene therapies is key for the clinical translation of advanced treatments that can target the molecular foundation of hereditary and acquired cardiac disorders. Here, we pursue the hypothesis that an advanced, scalable AAV purification methodology not only provides higher AAV yields than conventional approaches but generates ultra-pure vectors with superior biological and therapeutic capabilities.

Based on our current cardiovascular gene therapy programs, we established an affinity chromatography (AC)-based methodology for the scalable production and purification of AAV vectors to meet the needs of translational studies. Mass spectrometry analysis demonstrated that the conventional density gradient (DG) purification resulted in a highly contaminated vector/non-vector protein mix (AAV9 5.49% vs. 94,51% contaminants; 234 proteins) while AC delivered ultrapure AAV9 vectors (AAV9 93.24% vs. 6,76% contaminants; 3 proteins), further reflected by electron imaging and SDS-page analysis. Potential differences in biological potency of AC- and DG-purified AAV9 vectors towards cardiac transduction were determined by systemic injections of $1{\cdot}10^{10}$, $1{\cdot}10^{11}$ or $1{\cdot}10^{12}$ vgc of AAV9-EGFP in C57B6 mice (n=8 mice each group). AC-purified AAVs achieved a significantly higher cardiac transduction efficacy for every dosage assessed by comparative bulk myocardial DNA, RNA and protein level analysis after 2 weeks. Therapeutic potency was subsequently examined for a recently published novel target for chronic heart failure consisting of the exogenous relaxin (RLN) ligand activated RLN family peptide receptor 1 (RXFP1) system. To this end, a dosage of $5{\cdot}10^{11}$ vgcs of either DG- or AC-purified AAV9-RXFP1 vectors were systemically injected and the cardiac contractile performance increase was captured after 2 weeks in anesthetized mice of both groups by invasive catheter-based left ventricular (LV) pressure rates in the course of exogenous RXFP1 activation through an intravenous injection of a single dosage of RLN. Of note, 10 minutes after RLN administration, the rise in LV $+dp/dt_{max.}$ was already significantly greater in the AC- than the DC-treated group (AC: 13594+/-1972 vs. DC: 9822+/-801 mmHg/s; n=8 per group, $p<0.01$).

Our novel data clearly promote AC-based AAV purification (here AAV9) as a novel standard for cardiovascular basic and translational research. Due to a superior biological potency and overall purity of the AC-purified vectors, such AAVs are expected to achieve higher consistency in results and higher therapeutic effects in pre-clinical basic and translational studies even at lower vector dosages.

P344

A High Quality *minicircle* production scale up success story: AEX-HPLC in-process controls to guide upstream process modification

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Minicircle DNA production starts with the propagation of a parental plasmid (PP) molecule within a bacterial host strain. At the end of the cultivation phase, an *in vivo* recombination reaction is triggered that allows the *minicircle* (MC) region containing the Gene of Interest (GOI) to be separated as an independent supercoiled construct. The resulting mix of DNA molecules is called a recombination product (RP) which is taken through further proprietary purification stages in order to obtain the pure MC free of side products such as miniplasmid (MP).

Capillary Gel Electrophoresis (CGE) is a routine analytical tool to characterise the final purified MC product. Although CGE can be used to analyse RP, its applicability as an in-process control is limited to cases where the MC and MP have different sizes. When MC and MP are of similar sizes, one had to rely on agarose gel electrophoresis for the differentiation between the two molecules, wherein again a meaningful quantification based on band intensity is not possible. We employed Anion Exchange High Performance Liquid Chromatography as a second method to analyse one-step purified RP samples from our upstream department. AEX-HPLC could beautifully complement the data from CGE and was able to resolve the MP and MC of sizes 4.6 kb and 4.5 kb. Measuring the MC content in fermentation samples gave us unprecedented insight into RP composition and allowed us to make adjustments in the upstream process with the aim of maximising DNA yield per batch. Interestingly, we found that parameters such as induction timepoint and specific growth rate at induction notwithstanding, the proportion of MC in the RP was quite stable whereas the levels of PP and MP balanced each other out. Since only the total amount of these two contaminating molecules mattered in the downstream purification and not the individual amounts, variations in these two molecules could be followed without any consequence. The CGE data for proportion of PP correlated over the entirety of the production project with that from the HPLC data. The final purified MC DNA amount matched with the prediction from in-process controls (27% of RP). We achieved an overall scale up of MC production in *High Quality* grade of over 30X. To allow for possible losses in amount during purification, a large amount of starting material as RP was produced as a safety net. Nevertheless, the final MC product actually overshot the required amount by 3X.

Minicircles have proven benefits for enhancing the quality of AAV vectors in the context of the packaged genomes thanks to the elimination of false encapsidation of backbone sequences. Scaling up MC production will be the obvious step to cover the increasing demands of starting material for large scale AAV vector production while ensuring vector safety and quality according to regulatory guidelines. On the other hand, large scale production of *minicircles* in *High Quality* grade is also attractive for non-viral vector applications which are making a big comeback.

P345

Gain and loss-of-function screens identify targets with improved AAV production

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Recombinant AAV (rAAV) is a promising gene therapy vector, but its current manufacturing methods result in poor quality and yield. In this study we present three different approaches to improve rAAV production: modulation of signaling pathways using small molecules: A library of over 3000 bioactive small molecules was screened using the ATLAS platform to identify compounds that increase the capacity of cells to produce AAV9. A novel compound (SM-016) was identified that increased rAAV9 production, up to 3-fold, in a robust and dose-dependent manner. Engineering of clonal suspension-adapted HEK293 cells: A proprietary clonal suspension-adapted HEK293 cell line (AC001.230) was developed that shows improved productivity for 7 out of 10 tested serotypes compared to 293F cells. Through a targeted CRISPR/Cas9 screen, three classes of targets were identified that significantly increased AAV9 production compared to the wild-type clonal cell line. Two independent suspension-adapted knockout cell lines (AC003 and AC010) were developed that show greater than 2-fold improvement in AAV9 production capacity. The findings from these approaches have the potential to significantly reduce the cost of rAAV gene therapy, making it more widely accessible to patients.

P346

How to accelerate cell and gene therapy manufacturing from pre-clinical stage through clinical phases towards commercial production

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Over the past few years cell and gene therapy (CGT) has evolved from treating rare diseases to targeting prevalent diseases from cancer to diabetes to Parkinson's, leading to 27 marketing authorizations approved by the FDA and 11 approvals by EMA though 2022.

However, CGT production processes require special manufacturing considerations and often change from the initial concept in academic research settings to production under good manufacturing practices (GMP) in clinical and commercial facilities. Consequently, process development and optimization are required to adapt the initial academic process into a fully GMP compliant manufacturing process. This mandatory translation could be lengthy and costly, but new manufacturing approaches and technology have improved the process industrialization. We will review how emerging technologies can facilitate and accelerate the process development to arrive at a scalable GMP production process level. In particular, manufacturing considerations from requirement of end-to-end aseptic using single use technology, isolator solutions for closing off open manipulations, to process automation will be explored. Several factors will impact the process design including the following:

- Cell culture mode: adherent, suspension, and /or continuous culture
- Viral or non-viral transduction needed or not
- Production process equipment utilized
- Production modality and scale will impact the culture volumes and bioreactor style required.

This presentation will walk the audience through process design considerations and emerging technologies to enable clinical stage and commercial manufacturing of CGT products tailoring to a growing patient population.

P347

Comprehensive characterization of Mesenchymal Stromal Cells unveils features predictive of immune modulation capacity

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The functional characteristics of mesenchymal stromal cells (MSCs) vary significantly and the effectiveness of MSC-based therapeutics may be improved by identifying predictive features associated with a given function.

Upon priming with IFNg, MSCs undergo profound changes that enhance their immunomodulatory potency *in vitro*. In this study, we thoroughly characterized resting and primed clinical grade MSCs from adipose tissue and placental decidua, and correlated phenotypic, genetic and epigenetic features with their immunosuppressive capacity. The characterization included:

-Morphological analysis (ZEISS Celldiscoverer 7, high throughput data analysis on cell profiler software)

-Cytokine secretion (45 analytes on Human XL Cytokine Luminex® Performance Panel)

-Gene expression (25 immune modulatory genes on Quantstudio qPCR)

-miRNA profiling (800 human miRNAs on Nanostring® panel)

-Surfaceome (BioLegend® platform, 361 surface proteins).

Stimulated peripheral blood mononuclear cells (PBMCs) from four healthy donors were co-cultured with the MSCs and analyzed for proliferation and expression of activation markers. The MSC immune suppression capacity was measured by the % of CD4 and CD8 proliferation inhibition.

Adipose-derived stromal cells (ADSC) exhibited a better immune suppressive profile, compared to decidua-derived stromal cells (DSC).

Live-imaging followed by a high-throughput analysis of 88 morphological features revealed that morphological changes induced by IFNg are more pronounced in ADSC (26 cellular features and 1 nuclear feature in ADSC *vs* 4 cellular features in DSCs).

A Pearson correlation analysis identified that the changes in perimeter, Feret diameter, major axis length, compactness, and n-Zernike_6_4 of MSCs upon priming are highly correlated with immune suppressive capacity.

IFNγ induced the secretion of IL-10, IP-10, IL-17E, GM-CSF, FGF basic, TGF-a, MCP-1, IFNg, and IFNa. A higher VEGF secretion in the resting state was predictive of a better immune suppressive capacity.

The expression of immunomodulatory markers was significantly upregulated upon IFNg priming with a similar pattern for both ADSC and DSC, with IDO, CXCL9, CXCL11, DHLAG, and CXCL10 as top 5 upregulated markers. The immune suppression capacity positively correlated with the upregulation of CXCL11, LGALS9, IL7, CXCL9, DHLAG, VCAM1, IDO, PDL-1, IFIT1, FLT3, and negatively correlated with MCP3. Baseline increased gene expression of CD83, VEGFA, TRAIL, and FGF2 in resting MSCs was also associated with enhanced immune suppression.

Two hundred-one miRNAs were detected in ADSC and 329 in DSC. The upregulation of 10 miRNAs (hsa-miR-92a-3p, hsa-miR-301a-3p, hsa-miR-28-5p, hsa-miR-543, hsa-miR-1224-3p, hsa-miR-4516, hsa-miR-548a-5p, hsa-miR-29c-3p, hsa-miR-126-3p, hsa-miR-216a-5p) upon priming was significantly correlated with MSC immune suppressive function in both cell types.

IFNg priming induced the expression of surface markers implicated in immune modulation/activation (CD38, CD40, CD317), intercellular adhesion and migration (ICAM-1, integrin α3), and immunological checkpoints (CD274, HVEM). The increased expression of CD49F, CD274, CD54, and CD182 upon IFNg priming, and of CD140b, CD26, TSLPR, CD36, MUC-13, CD213a2, Notch3, and Tim-4 on resting MSCs, significantly correlated with immunomodulatory capacity.

A major limitation for large scale manufacturing and clinical application of MSCs is represented by the lack of standardization and potency variability. Through an extensive characterization of the clinical grade products, we identified factors that can guide selection and manufacturing of MSC products with enhanced immune suppressive potency.

P348

Production of synthetic AAV genomes from *minicircle* templates for enhanced gene therapy applications

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We had previously presented several fundamentally different approaches aimed at increasing the efficiency and homogeneity of rAAV production. One of the aspects we looked at was *in vitro* production of AAV genomes, termed Aavecule® DNA, for use in AAV production and other gene therapy applications, like *in vitro* AAV assembly and lipid nanoparticle-based delivery. Our *in vitro* method of synthetic AAV genome generation has two main advantages: Firstly, the backbone

sequences are removed even before they encounter the replication and packaging functions in the producer cell line, thereby drastically reducing the probability of their occurrence in the recombinant vector particles. The AAV-like genome can be transfected into the producer cell line in a form that is already well suited to directly initiate the packaging reaction without having to undergo rescue from a circular substrate first. These synthetic genomes exhibit a structure that is quite similar to the one that would be encountered by the cell following a wild-type AAV infection. Secondly, this offers the possibility to move to a completely *in vitro* process for AAV production, where artificially assembled capsid proteins interact with synthetic AAV genomes to drive packaging. Encapsulation of the synthetic genomes in lipid nanoparticles is another exciting non-viral and scalable possibility for gene transfer with the potential to facilitate patient access to these vital therapies. We emphasized the ITR sequence integrity and a laid special focus on monitoring this critical vector quality parameter.

We present here the applicability of *minicircle* DNA as templates for the synthetic AAV genome production, which is based on Rolling Circle Amplification. These *minicircles* are derivatives of an ITR-transgene-plasmid wherein the bacterial backbone sequences have been removed through an *in vivo* recombination step. The *minicircles* were then purified to remove all traces of the original plasmid or any recombination side-products resulting in a highly homogeneous, monomeric preparation. These molecules are produced in *High Quality* grade, meaning they are suitable to be used not only for pre-clinical studies but even for future applications involving clinical phase studies. We used a *minicircle* template containing ITR sequences and a GFP reporter gene as genetic cargo as the base material for synthetic AAV genome production. Such *minicircles* have been previously proven to be effective in producing AAV particles of superior purity when compared with plasmids. In the resulting synthetic AAV genome, the *minicircle* scar sequence directly adjoining the ITRs, is removed, giving a completely synthetic starting material for manufacturing the highest quality gene therapy products.

P350

Enhancing adenovirus gene transfer using novel nanoparticle complexes

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Over the past few decades, gene therapy has become a revolutionary strategy for treatment of genetic diseases and vaccinations using viral vectors and nucleic acids. Important to this, is consideration of cost of vector production to meet expected future worldwide demand and improving gene transfer efficacy and safety. Among viral vector, adenoviruses (Ad) are commonly used for their versatility especially for vaccination. However, they are still associated with cytotoxicity.

In this study, we sought to improve Ad transduction to reduce dose and associated cytotoxicity using novel Nuvec® silica nanoparticles (SiNPs). Nuvec® SiNPs are typically coated with low concentrations of PEI. However, here we compared Nuvec®/PEI and PEI free Nuvec® SiNPs when complexed with Ad to determine the functional differences in surface topography that determine cytotoxicity and gene transfer efficacy. Nuvec®/PEI and PEI free Nuvec® complexed with Ad were compared to Nuvec® free Ad in infecting liver, breast cancer cells and primary muscle cells, as representative cells targeted by gene therapy of cancer and vaccination,

respectively. Gene transfer efficiency was measured by quantification of β-galactosidase reporter gene expressed by Ad and cell toxicity was analysed using trypan blue exclusion.

Nuvec® or Nuvec®/PEI were used at a range of concentrations between 2-160ug/ml complexed with Ad at different MOIs. Increased viral infectivity was observed with both Nuvec®/PEI and PEI free Nuvec® Ad complexes compared to Ad alone for all cell lines. However, Nuvec®/PEI outperformed PEI free Nuvec®/Ad gene transfer. In breast cancer cells a two-fold increase in transduction occurred with 2ug/ml of Nuvec®/PEI/Ad compared to Ad alone at the same titre, whilst PEI free Nuvec®/Ad was required at 80ug/ml to reach the same level of transduction of these cells. For liver cells, 20ug/ml PEI/Nuvec®/Ad doubled vector transduction efficiency of cells compared to PEI free Nuvec®/Ad. Muscle cells appeared poorly transduced by Ad alone, however, when complexed with PEI/Nuvec®/Ad up to ten-fold increase in gene transfer was observed using 80ug/ml nanoparticles. Overall, by reducing Ad dose, interestingly, PEI/Nuvec® enhanced Ad gene transfer at the same level of Ad used at doses up to 20 times higher.

We next investigated whether improved Ad gene transfer by complexing with PEI/Nuvec® could also provide vector protection to storage at different temperatures. When complexed with Nuvec®/PEI and incubated at room temperature or at 4° for 14 days, Ad particles retained their infectivity of liver cells up to 80% and 40%, respectively, in contrast to infection with Ad alone which decreased by 70% if incubated at 4° and complete loss of infectivity after room temperature incubation for this period.

We conclude PEI coated Nuvec® provides significantly enhanced Ad transduction to enable effective gene transfer at lower MOI, thereby reducing the amount of vector required for therapy and with reduced unwanted cytotoxic side effects. Furthermore, PEI coated Nuvec® improves the storage conditions required by Ad particles, which reduces waste and loss of particle viability during transit between laboratories and the clinic.

P351

Designing, building and commissioning a multi-product Cell and Gene Therapy clean room facility: lessons learned.

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1: The Netherlands Center for the Clinical Advancement of Stem Cell and Gene Therapies

The Netherlands Center for the Clinical Advancement of Stem Cell and Gene Therapies (NecstGen) is a Center of Excellence and CDMO in the Netherlands. Entirely funded by a hospital center, university, and government, NecstGen completed the building process of a 4,000 m^2, state-of-the-art cleanroom facility consisting of 13 grade B and grade C cleanrooms. To fulfill NecstGen's mission the facility must be appropriate to handle both Cell Therapies and viral vector-based Gene Therapies for many years to come.

When designing and building a clean room facility, critical decisions must be made requiring different areas of expertise; not only specialists in engineering and construction but also manufacturing and quality as part of the design team. The NecstGen facility is constructed in an existing building. This had the advantage of reducing contamination risks because the building was already dry and had low humidity levels, but brought with it the challenges of dealing with existing dimensions, load-bearing walls, floor strength limitations and infrastructure.

During design, optimising the floor plans led to a 50% increase in usable cleanroom space compared to the initial drawings. The facility is designed with segregated HVAC for all clean rooms, full temperature/humidity control, integrated particle counters, building and equipment monitoring systems to allow full control of all areas. Electrical power is secured through an uninterrupted power supply and an emergency power generator. The unidirectional flow principle is applied to separate flows for incoming/outgoing operators, starting materials, consumables, final products, and waste.

Building a brand-new facility also required additional activities, such as qualification of the facility and equipment, setting up a quality management system, and building a brand-new team of experts. This necessitated detailed planning and alignment of all activities to be completed before the regulatory GMP inspection.

After a design and engineering period of 7 months and a construction period of 18 months, the facility was ready. The NecstGen facility had passed all performance qualifications and was ready for GMP certification. NecstGen obtained it's GMP license in December 2022.

Here NecstGen aims to share lessons learned along the journey of facility completion.

P352

Assessing the underlying cellular response of HEK293 cells to evolutionary pressures

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Human embryonic kidney 293 (HEK293) cells have been established as a suitable expression system for the manufacturing of viral gene therapy vectors, such as Adeno-associated virus (AAV). Despite the growing demand in industrial applications, limited efforts have been made to understand the cellular response to long-time evolutionary pressures and/or production associated stresses; critical information to improve product quality and/or titer by cell line and process development. As such, this study aims to enhance the understanding of these mechanisms, by assessing the genetic and epigenetic variability of HEK293 cell lines to evolutionary pressures (e.g. adaptation procedures). As a first step, HEK293 cells were adapted to suspension growth, using several commercially available media formulations. Adapted cells were then phenotypically characterized (e.g. cell growth and productivity) and compared to reference suspension cell lines, as well as their adherent growing parent. Finally, whole-genome and DNA-methylome analysis will be performed, assessing the underlying biological mechanisms of this adaptation procedure. Overall, this study highlights new insight into the response of HEK293 cells to evolutionary pressures and can be considered as the basis for further omics characterization, that can be leveraged to engineer HEK293 cells towards an improved production performance.

P353

A concept of decentralized point-of care manufacturing of cell and gene therapy – technical and regulatory considerations

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The success of personalized cell or gene therapy (CGT) reflects a growing demand for personalized medicine that is accessible and affordable. The number of potential CGT therapies for conditions ranging from hereditary disorders and autoimmune diseases to cancer and diabetes is growing exponentially. In this presentation a model for decentralized manufacturing at point of care will be presented.

Manufacturing autologous therapies could occur in one of two settings: regional facilities managed by developers or across certified treatment delivery centers (e.g., academic health centers), closer to the patient's bedside (similar to the way stem cell transplants are managed currently). The latter option is dependent on establishing closed-system manufacturing that minimizes the infrastructure requirements at treatment facilities and maintains compliance with regulatory requirements and quality standards. The interdependence of such centers in a 'hub-and-spoke' network has the potential to make manufacturing resilient and largely immune to cold-chain disasters and fluctuations in demand.

The production of therapies must be carried out in a strictly controlled and monitored environment according to drug manufacturing for therapeutic purposes. To ensure the scalability of the decentralized model but also the harmonization of the product quality, it is necessary to operate in a standardized environment.

Orgenesis has designed and developed a production framework according to applicable international quality standards. It has transformed the traditional fixed ("brick and mortar") production room into a mobile, harmonized unit: the Orgenesis Mobile Processing Unit and Lab (OMPUL) that can easily be deployed throughout a POCare Network. OMPULs are designed to enable parallel processing of CGT products in a safe, reliable, and cost-effective manner at the point of care.

A comprehensive approach is proposed addressing "capacity crunch" by establishing regional POCare centers utilizing the above described Orgenesis Mobile Processing Units & Lab (OMPUL) solution, providing similar manufacturing environments, while enabling flexible localization of processing units to minimize logistic complexity and enable expedited scale out. The POCare platform is supported by a methodology that is based on present regulatory thinking. Adherence to good manufacturing practice (GMP) standards while assuring quality control and harmonization across the POCare network of facilities is performed by establishing a Control Site that serves as the primary focus of regulatory interface.

In general, the Central and Regional QMS support GMP oversight, while the Control Site addresses CGT specific related issues, e.g., change to approved manufacturing/testing process or product related deviation. The Control Site is proposed to be the primary focus of regulatory oversight, focus point for interaction with regulatory agencies, and provision of systems implemented for overall quality assurance (e.g., incidents, Out of Specification or compliance events, serious breaches, change control and periodic audit of systems and sites). The Control Site takes responsibility of the POCare platform once the product is ready for decentralized manufacturing, i.e., the tech transfer considered as complete and successful.

P354

Evaluation of several process cell lysis reagents as replacements for Triton X-100 for rAAV production

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Triton X-100 is a detergent used to lyse production cells and release AAVs during rAAV manufacturing processes, which also has a complementary activity of detergent viral inactivation on enveloped adventitious viral contaminants. 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).

In this work, we have evaluated four different cell lysis reagents for rAAV production and also for viral detergent inactivation properties. For cell lysis, results show that the required duration of cell lysis (10, 30, 60, 90 and 150 min) is different for each lysis reagent, but after 150min of incubation, a comparable rAAV production is reached for all agents. Secondly, the lysis reagents do not have similar efficiency as regards adventitious viral inactivation compared to Triton X-100. As previously shown by others, we have confirmed that Polysorbate-type reagents are sufficient to lyse cells, but not to inactivate enveloped viral particles and it is necessary to add a solvent such as TnBP, which is already used for viral inactivation in plasma products, to ensure viral inactivation.

Regarding our results, a new detergent, Simulsol (SL-11W) seems to be promising for both cell lysis and adventitious viral inactivation, and has the advantage that an additional solvent is not required to be added for adventitious viral inactivation effects. Whereas Triton X-100 and SL-11W have distinct membrane-disruptive effects in terms of their mechanisms of action, we have confirmed SL11-W effects cells lysis during the production of rAAV2/8 and rAAV2/9 vectors (bulk harvest) and have also demonstrated its efficacy for viral inactivation.

In conclusion, different polysorbate detergents demonstrate different properties (required incubation times) for AAV process cell lysis and require additional solvents if adventitious viral agent inactivation is also desired, and SL-11W is a potential candidate to replace Triton X-100 for rAAV production.

P355

Increased viral vector yield: Development and scale-up validation of Small Molecule Enhancers

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Manufacturing of gene and cell therapy vectors is a complex process, often resulting in insufficient manufacturing yields to satisfy developmental and commercial demand. While the last decade has seen major advances in viral platform, bioreactor design, cell line and media formulation optimization, the industry still faces major challenges with production consistency and yield.

An often overlooked and underappreciated bottleneck in cell culture-based manufacturing is the presence of cellular innate antiviral immune pathways that remain partially, if not fully, intact in producer cells. These well-conserved pathways are triggered via the detection of foreign nucleic acid and/or molecular bi-products of viral replication/assembly resulting in blunted viral vector yields in both infection and transfection-based manufacturing strategies.

Virica's Viral Sensitizers (VSEs™) encompass a proprietary collection of small molecules that enhance the growth of viruses by transiently and efficiently dampening cellular antiviral defenses. Leveraging high-throughput methods, Virica has assembled a library of over 130 small molecules which enhance viral production by transiently antagonizing a broad range of cellular innate antiviral pathways. Owing to different molecular mechanisms, VSEs™ can be combined and formulated for specific uses across various viral and cellular platforms.

Here we describe the development of custom small molecule formulations for enhanced production of therapeutically relevant vectors including AAV (Adeno-Associated Virus), using high-throughput methodologies.

Furthermore, we present validation data supporting the scalability of AAV-specific custom formulations yielding multi-fold increases in genomic titer, capsid titer, as well as full to empty ratio in formats representative of large-scale commercial platforms including the Ambr®15 microbioreactor system and bioreactor systems up to 5L working volume.

Ultimately, this data supports the broad applicability of small molecule enhancers, such as Virica's VSEs™, and their use as a novel solution for increased production of viral vectors at scale.

P356

A roadmap towards effective and accessible adoptive T cell manufacturing

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Cell and Gene Therapies have the potential to provide a cure for thus far uncurable diseases. Although very promising, the manufacturing of living cells as a therapy brings its specific challenges including cost and related patient access. As the field of Cell and Gene Therapy is currently still young, manufacturing methods often involve open processes requiring grade B clean rooms although a trend towards the use of closed processes and bioreactors are emerging. Each cell type has its own requirements with respect to culture conditions, medium additives, and cell densities.

Process development aims to translate a research-grade process into a GMP-compliant manufacture method, which is robust and reproducible despite the donor-to-donor variations. During process development, it is important to identify the critical process steps and the critical quality attributes and to develop adequate analytical assays and potency tests. However, at this stage key decisions should be made, and activities can be undertaken to challenge the cost of goods of a therapy.

Manufacture of adoptive T cell therapies, such as TCR and Car T cells, includes a selection of starting population, cell activation, transduction, expansion and fill and finish of the product.

During process development, many decisions have to be made on raw material usage, transduction method, cell expansion methods, use of bioreactors, and quality control testing.

Here we aim to share our knowledge on process development and testing strategies for adoptive T-cell therapies by providing an overview of the pros and cons of the different process choices. Including automation not only of the process but also quality control and novel release assays.

P357

Optimization and Scale-up of AAV Manufacturing Using a cGMP Lipid Polymer Nanocomplexes (LPNCs) and Small Molecule Enhancer.

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The current development of new gene therapies designed to treat a range of diseases is growing rapidly due to clinical successes, this has led to an exponential increase in the number of registered clinical trials recruiting recombinant adeno-associated virus (AAV) for gene therapy. At the forefront of these clinical trial efforts, efficient viral vector production for manufacturing by transient transfection is in high demand to ensure maximum yields and reduced costs. With the aim of supporting scale-up AAV manufacturing platform technologies, Mirus Bio researched and developed innovative transfection solutions with simplified, cost-effective workflows that offer high titers of viral genomes. Mirus transfection technologies consist of unique mixtures of lipid and polymer that enable the formation of lipid polymer nano complexes (LPNCs) which can result in a 2-10-fold increase over existing technologies. To further support this production, we developed a small molecule enhancer that can enhance upstream AAV production. During the development of the reagent and enhancer, we studied important characteristics to ensure scalability, effortless optimization, and smooth adoption within AAV manufacturing which are presented here. We also evaluated and optimized the small molecule enhancer with the transfection reagent at a benchtop bioreactor scale. The combination of transfection reagent and optimized enhancer solution were studied across a range of suspension HEK 293 subtypes in bioreactors and measured by viral genomes and the percentage of full capsids. Additional tests were performed to show variable amounts of pDNA. We also show they can be used to produce multiple AAV serotypes including AAV2, AAV5, AAV8, and AAV9, with measured AAV titer and virus quality. Overall, our data demonstrate that our unique transfection solution, *Trans*IT-VirusGEN® Transfection Reagent together with the *Rev*IT® small-molecule enhancer can produce high titers of AAV at the benchtop bioreactor scale which is ideal for cell and gene therapy developers by consolidating high performance and scalability to support cost-effective manufacturing to support more patient doses per run.

P358

Development and scalability of a robust suspension-based production process for AAV9

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Recombinant adeno-associated virus (rAAV) is among the most widely used viral vectors for gene therapy. A broad range of different capsids have been used in clinical trials with AAV9, 8, 2 and 5 being the most prominent serotypes applied in that order. AAV manufacturing processes typically comprise platform modules which work universally across serotypes, whereas other steps require specific development and optimization for a given serotype or capsid variant such as molecular plasmid design, plasmid ratios, affinity and full/empty chromatography.

We have developed a robust suspension platform process based on our proprietary HEK293 cell line, that is optimized towards yield at best possible quality with full scalability. The platform is proven for several capsid serotypes and vector genome sizes. We are continuously expanding our data to show universal applicability of the platform.

We present here the development and scalability of a robust suspension-based production process for AAV9. Using our proprietary split 2-plasmid system we applied a screening strategy on the Ambr® 15 bioreactor system to determine the best parameters for AAV9 production. The most promising conditions were then tested and validated in scaled-up bioreactors representative of large-scale production formats. A broad range of analytical methods were applied demonstrating comparable yields to already proven rAAV references at best possible quality.

The data presented here demonstrate the successful development and scalability of a suspension-based production process for AAV9 illustrating the capabilities of the established suspension production platform for rAAV manufacturing.

P359

Scalable continuous flow Iodixanol gradient ultracentrifugation purification of supernatant derived rAAV8 with enhanced purity when compared to batch CsCl gradient ultracentrifugation.

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1: InnovaVector SRL 2: Alfa Wassermann B.V

Adeno-associated virus (AAV) is one of the most promising gene therapy vectors due to their low immunogenicity and large tropism. A robust, reliable and scalable purification process for AAV vectors is essential to the gene therapy industry. AAV purification techniques can be separated into two distinct categories, moreover density gradient centrifugation or chromatography purification. For the serotype-independent purification of AAV vectors produced in HEK-293 or SF9 cells, the two most widely established methods for preclinical applications are based on batch

ultracentrifugation using either cesium chloride (CsCl) or an Iodixanol density gradient. Iodixanol is inert and non-toxic to mammalian cells, and should be favored for scaling up. In addition, Iodixanol has sufficient viscosity to form a stable gradient. Reported data suggest that vectors based on many commonly used AAV serotypes are present at high titer in culture medium of transfected cultures, and can be recovered simply by harvesting and concentrating the culture fluids. Here, we report a simplified and scalable rAAV8 vector manufacturing process using supernatants of triply transfected HEK 293 cultures and PEG precipitation strategy. To this end, we compared the final product profiles obtained : 1) via 2 rounds of CsCl gradient-based batch ultracentrifugation and 2) 1 round of Iodixanol gradient-based continuous flow zonal ultracentrifugation. Data obtained showed the presence of a significant quantity of rAAV8 viral particles in the supernatants of the cell cultures. This quantity is similar to one that can be obtained after cell lysis. A significant improvement of impurities profile (decrease of residual total host cell protein and cellular DNA) in the final product has been observed after purification through Iodixanol gradient performed using continuous flow ultracentrifugation with respect to the one obtained after cesium chloride gradient batch ultracentrifugation while total particles recovery (process efficiency) and vector purity are similar in both purification processes. Due to its high capacity and scalability these data suggest that supernatant derived from rAAVs purified with one round of Iodixanol gradient-based ultracentrifugation could be a suitable process for producing enough material for clinical application in cost and time effective way.

P360

doggybone DNA (dbDNA™): An advanced platform for genetic medicines

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Demand for DNA has risen dramatically in recent years, driven by significant growth in the clinical development of cell and gene therapies. The supply of plasmid DNA has become a major bottle neck in the ever-expanding genetic medicine sector; new, scalable, faster DNA production technologies are vital for this sector going forward.Touchlight's enzymatic DNA amplification technologies offer 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. The proprietary technology developed for high-scale DNA production is based on rolling circle amplification (RCA), allowing for the generation of large quantities of high-quality GMP DNA. The ability of this procedure to generate highly purified, bacterial-sequence free dbDNA products offers advantages over other conventional and 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. Such advanced technologies of manufacture should address a well-characterisedbottleneck in DNA production capacity.

P361

Ultra-Cold Storage of Lentiviral and Adenoviral Vectors in Cyclic Olefin Polymer Containers

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ABSTRACT OVERVIEW

Adenoviral (AdV) and Lentiviral (LV) vectors are major platforms for developing vaccines and cell and gene therapies. Ultra-cold storage of the viral vectors can be helpful to ensure uninterrupted supply of vaccines in critical times such as a pandemic and in the case of off-the-shelf oncologic and advanced therapies.

Polypropylene (PP) vials and tubes have been commonly used as the primary container in research, but they may be not suitable for clinical manufacturing of drug products, due to concerns related to container closure integrity of the snap cap- or screw cap-based systems and high particle generation from the material. Cyclic olefin polymer (COP) vials may be helpful to overcome these challenges and maintain better drug safety, purity and efficacy.

2 mL COP (Daikyo Crystal Zenith®) vials with bromobutyl serum stoppers and aluminum seals were compared to 1.8 mL PP screw cap vials and evaluated for the ultra-cold storage of Ad5-CMV-eGFP and Ad5.F35-CMV-eGFP viral vectors for 1 to 14 days. In a separate experiment, the vials were compared for the functional recovery of Lenti-GFP viral vectors, imitating a short-term storage condition and addressing potential concerns related to pH excursions upon exposure to and after removal from a dry ice-based shipping condition.

LEARNING OBJECTIVES

- AdV physical recovery was higher from COP vials than PP vials particularly upon shorter terms of ultra-cold storage. In either vial types, functional recovery was comparable to fresh sample after 1 day but declined before 7 days.

- LV functional recovery was comparable between COP and PP vials, in ultra-low freezer or dry ice. There was significant functional titer loss after filling and immediately dispensing from the PP vials, whereas there was no significant loss from COP vials.

- COP vials can be a suitable alternative to PP vials for the cold chain containment of the growing generation of viral vector-based therapeutics.

P362

A small-scale perfusion based hollow-fiber bioreactor supports a scalable T cell manufacturing process

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The Quantum Flex® Cell Expansion System is the next generation of hollow-fiber bioreactor-based technology for automated, functionally-closed, scalable cell expansion which features two bioreactor sizes to choose from: standard and small. Utilization of the small bioreactor allows scaling of starting material and reagent usage relative to the standard bioreactor. For this data set, cells from a single donor were expanded in the small bioreactor where cell seeding number and feed rates were scaled to 20% of those typically used in the standard bioreactor. The small bioreactors were seeded at 6 million T cells, cultured for 8 days, and yielded 2.4 to 3 billion cells (N=4). Cells displayed high viability, low exhaustion, and desired phenotypes such as T_{cm} and T_{scm}. Moreover, new disposables allow for unique media handling strategies for a reduction in reagent and media usage. Less than 1.5 liters of complete media was used in the small bioreactor expansions. Quantum Flex® can be used to support any stage of the cell manufacturing process from process development, single dose generation starting with few cells, scaling up to standard bioreactor to support multi-dose manufacturing, and even scaling out by managing a fleet of devices via Quantum Flex®'s cell processing application software, all while keeping the expansion platform and core technology consistent.

P363

Developing novel Droplet Digital PCR methods for the quantification of gene and cell therapy targets

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Droplet Digital Polymerase Chain Reaction (ddPCR) technology allows PCR to be performed on a multitude of compartmentalised reactions simultaneously, giving positive or negative results for each droplet, and thus increasing assay sensitivity and accuracy in comparison with methods such as quantitative PCR (qPCR). A standard curve is not required for this direct quantification, improving set up efficiency and data accuracy. Furthermore, the sample partitioning also affords the assay an increased tolerance to the potential effects of PCR inhibitors existing within a range of biological sample types.

We have therefore established a ddPCR testing platform to allow for DNA or RNA targets to be accurately and efficiently quantified, even when the expected copy number is low. We have developed methods for assessing a range of DNA or RNA targets (with an additional reverse transcription step), which can then be validated to Good Laboratory Practice (GLP) standards to allow for sample analysis in regulatory studies. One example is an assay that we developed to detect and quantify a vector DNA target in mouse blood and tissues following administration of Chimeric Antigen Receptor (CAR)- T cell therapy.

Here we present data obtained during the development process for these assays and give an insight into the potential use of ddPCR in furthering our capacity to accurately quantify nucleic acid targets as part of the development of new gene and cell therapies.

P364

Validation of an analytical capillary gel electrophoresis method for the relative quantification of plasmid DNA topoisoforms

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Plasmid DNA serves as starting material to manufacture many advanced therapy medicinal products based on adeno-associated and lentiviral vectors and mRNA. One critical quality attribute during plasmid production is the plasmid topology. The desired plasmid topoisoform is covalently closed circular (ccc) as it features superior biological activity and transfection efficiency compared to the open circular (oc) form. Therefore, plasmid production aims at achieving high purity of the ccc form by specific removal of the oc form.

While agarose gel electrophoresis and densitometry are still employed as a classical method for the assessment of plasmid DNA quality for in-process controls and final DNA products, capillary gel electrophoresis (CGE) is the quality control method of choice for the precise determination of ccc and oc topoisoform relative quantities. The validation of analytical procedures according to ICH guideline Q2 constitutes a regulatory requirement in the GMP compliant area and also demonstrates credibility of a method in non-GMP manufacturing processes.

Based on our extensive experience in plasmid characterisation by CGE and from preliminary tests, a validation plan was designed and experiments were carried out accordingly, using the in-stock plasmid PF463 as test item. The validation covered the parameters linearity, accuracy, repeatability, intermediate precision and specificity. Linearity was demonstrated in a range of 50 ng/mL to 160 ng/mL, with constant ccc form percentage across the whole range, resulting in a coefficient of variation (CV) of 0.47 %. Due to unavailability of an authentic reference material and difficulty of plasmid topoisoform spiking studies, accuracy was shown by calculating the recovery against the linear regression curve. The method was proven to be precise with CVs of 1.01 % for repeatability and 1.26 % for intermediate precision. In the presence of several spiked impurities, the peaks of the plasmid topoisoforms could still be separated, demonstrating the specificity of the method. This validation demonstrated that the analytical procedure is suitable for its intended purpose to separate and determine relative quantities of plasmid DNA topoisoforms.

P365

Quantitative determination of host cell RNA residues against a DNA background by LC-MS

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The quality considerations for plasmid DNA starting materials to be used in the manufacturing of viral and non-viral gene therapy vectors is a constantly evolving area. Along with the rapid advance of the field gene therapy, the onus is on the manufacturers to ensure a supply of starting materials of reliable and reproducible quality. Critical contaminants from the plasmid DNA production process have been identified by regulatory agencies. Especially, the DNA products

used for the manufacture of mRNA vaccines or ATMPs require a starting material / drug product free of bacterial RNA residues. According to the FDA guidelines, a maximum of 1% by weight of host RNA in DNA should not be exceeded. Usually, the absence of RNA in final plasmid DNA products has been ascertained through visualization by agarose gel electrophoresis, which is limited in its capacity for accurate quantification through densitometry. We describe here a powerful new method to selectively generate nucleotides from RNA against a DNA background, separate these and quantify a target nucleotide through Liquid Chromatography – Mass Spectrometry (LC-MS).

We isolated samples through a typical plasmid capture step: cleared lysate from bacterial cells, flowthrough from a chromatography, elution from the column and finally fully purified plasmid DNA products of final adjusted concentration. RNA and DNA nucleotides in the form of their sodium salts were used to establish corresponding standard curves. Following hydrolysis, samples were separated on a UPLC-system using a normal phase column. Finally mass spectrometric analysis was performed on a triple quadrupole MS instrument.

Calibration curves for four ribonucleotides and two deoxyribonucleotides were established. Through the hydrolysis of a control RNA as well as DNA sample parallel to the chosen ribonucleotide and deoxyribonucleotide analytes, it was observed that we could selectively hydrolyse RNA whereas DNA remained intact.

Quantitative analysis of host cell RNA residues in plasmid DNA purification samples was successful. After establishing the baseline value in the cleared lysate, we found that most of the RNA contamination is being removed in the flowthrough during the DNA capture step. Following weak binding conditions, our agarose gel electrophoresis suggested that RNA had bound to the column and eluted along with the plasmid DNA. The LC-MS data could nicely correlate with this observation. When using optimal binding conditions, the electrophoresis showed pure plasmid elution peaks which were again confirmed by the LC-MS data where no signals for the target ribonucleotides above the LOD could be detected. Similarly, the final pure plasmid DNA samples returned signals for RNA that were also below the LOD of the current method. The LOD is currently at 13 $ng \cdot \mu L^{-1}$ which, when taken together with our plasmid concentration of 1 $mg \cdot mL^{-1}$, correlates very well with the regulatory specifications. Further improvements in the method to decrease the LOD are underway.

P366

Optimizing an AAV downstream process with single-pass TFF

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Recombinant adeno-associated virus (rAAV) vector is widely used to deliver therapeutic transgenes for *in vivo* gene therapy. Transient transfection is the most common method for preclinical and clinical-grade rAAV manufacturing. TaRGeT's AAV manufacturing platform is based on transient transfection of suspension HEK 293 cells and delivers consistently high productivity of various serotypes.

The downstream process aims to concentrate viral vectors and to remove process- and product-related impurities. The classical workflow of AAV downstream process involves several

steps: clarification, concentration, affinity chromatography, ion exchange chromatography, concentration, and final sterile filtration. Success is highly dependent on using the right technologies to ensure an optimized, efficient, and predictable process.

The concentration step prior to capture chromatography typically involves tangential flow filtration (TFF) including a recirculation loop through cassettes or hollow fibers. In contrast to traditional TFF, the Cadence™ single-pass tangential flow filtration module allows direct flow-through concentration with no recirculation of product, which is time- and cost-saving and enables high concentration. AAV viral vector is particularly well-suited to single-pass TFF technology, which requires a longer fluidic path and a higher pressure.

Process development aimed to define the optimal concentration factor that enabled significant concentration to reduce loading time and therefore cost, while meeting the loading requirements of affinity chromatography. A panel of analytical methods was used to deeply characterize the product generated.

After clarification of AAV6 bulk harvest through depth filters from Cytiva, the single-pass TFF technology allowed a significantly lower feed volume of the capture step with high yield.

The successful development performed at 2L scale allowed a direct scaling-up to 50L scale with a single-use stirred tank bioreactor. A high concentration factor coupled with a good recovery was observed.

Implementing single-use Cadence™ single-pass tangential flow filtration module, which is well-suited for intensified performance of chromatography and connected and continuous processes, is expected to help meet the challenges of next-generation rAAV manufacturing processes.

P367

Quality over quantity: where is the trade off?

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One of the biggest challenges in rAAV manufacturing is tailoring the AAV production platform in such a manner to improve virus productivity, and simultaneously to uniformly improve a multitude of critical quality attributes, to meet both regulatory and commercial demands. The final design of the process should aim at high productivity with a higher percentage of full-length genome packed viruses, high vector potency but also with lowest possible impurity levels (mispackaged plasmid derived and host cell derived impurities).

Our platform is an end-to-end process solution that is differentiated from the industry. This process platform was optimized for yield at best possible quality, to align with the rigorous regulatory constraints that are continuously evolving while allowing for flexibility and adaption to specific customer program needs. This platform is fully scalable and proven for a number of capsid serotypes, based on HEK293 transient transfection using our proprietary split plasmid system.

Transient transfection is widely used to produce rAAV as it enables the agility and speed from gene to GMP typically being a major focus for product development companies. Besides cell line choice and media composition, plasmid design and the transfection procedure used are one of the

main critical process steps in upstream processing and are the major “driving force” for high quality and high potency product formation. The main goal of our study was to compare two transfection reagents currently available on the market and widely used for AAV manufacturing, and to determine the impact on the final product quantity and quality, as part of our continuous platform improvement strategy.

The transfection reagent that resulted in higher rAAV vector yields came with the disadvantage of changes in cell behaviour post transfection, indicating more rapid cell death most likely linked to the observed increases of undesired packaged impurities and overall worsening of vector quality. Furthermore, we continued investigating this phenomenon by optimizing the transfection conditions and we report here our observation regarding the impact this might have on the quality of the produced AAVs.

We are continuously challenging and optimizing our platform process, and while doing so, we always keep a quality mindset. Our aim is the production of the highest quality AAVs, that are scalable and feasible for manufacturing at a commercial scale, to support our clients and patients with the safest possible products.

P368

Automation of cold chain infrastructure provides benefits to time efficiencies, documentation rigor, and sample quality for cryogenic sample management workflows

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Automation of the various aspects of manufacturing is a critical component in process development for advanced therapies. Efforts to date have largely ignored cold chain infrastructure as automated solutions are often viewed as an expensive alternative to manual solutions. However, groups who have chosen to automate this aspect of their processes are demonstrating real benefits in the form of time efficiencies and process rigor. Moreover, regulators are increasingly looking for tighter controls regarding safety and security over cold chain compliance for managing therapies. Where products require controlled temperature conditions during transport and storage, temperatures should be tracked and recorded to confirm required conditions have been met. Any deviation from storage, logistical, and chain of custody requirements may have detrimental effects on preservation, viability, and patient safety. This study will quantify the impact of using cryogenic storage automation produced by Azenta Life Sciences as compared to a manual solution in the form of time savings and sample quality after one month of storage. Time requirements will be captured and reported for each method of storage and the samples will be examined and compared post-thaw for differences in morphology indicative of repeated freeze-thaw cycles. Such gains in time, data accuracy, and sample quality in cold chain infrastructure can be realized, and a considered a crucial benefit of investment in automation.

P369

Establishment of a model process platform for the downstream purification of enveloped virus-like particles to optimize host cell DNA and extracellular vesicle depletion

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Membrane-enveloped virus-derived particles offer various applications as vaccines and vehicles for gene therapy. Both, viral vaccines and gene therapy applications require a high degree of particle purity as defined by regulatory authorities. However, due to the novelty of these therapeutic approaches, there is currently no consent on a downstream process that fulfills these criteria for the purification of viral therapeutics. In addition, the challenging boundary conditions associated with many purification strategies are known to complicate the analysis of inevitable contaminants such as host cell DNA and extracellular vesicles (EVs). For example, DNA quantification methods are often affected by other contaminants such as RNA, proteins and high salt concentrations. Likewise, EVs have similar characteristics to virus particles, making it difficult to distinguish between the two. Another problem is that enveloped virus particles are fragile and require mild downstream conditions and a process time that is as short as possible.

Virus-like particles (VLPs) lack the virus-specific genome and are thus non-infectious while retaining most of the morphological properties of the donor virus. Combining high immunogenicity with cost-effective large-scale production, VLPs are promising vaccine candidates.

We are using fluorescently labeled human immunodeficiency virus (HIV)-derived VLPs as a model for enveloped viruses such as the lentivirus to accelerate the establishment of a downstream process through easier handling and production of the VLPs. As a first step, we are evaluating simplified approaches for contaminant detection and VLP quantification using fluorescence-based methods. Next, different membrane chromatography and filtration methods are implemented to optimize the separation of impurities, focusing on host cell DNA, and EVs. The results will be used to establish a scalable downstream processing platform for enveloped virus-derived vector particles and VLPs.

P370

Implementing AEX-HPLC as an orthogonal analytical method during plasmid DNA production

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Plasmid topology is measured to define homogeneity as a critical quality attribute during plasmid production. The desired form is the supercoiled conformation of a covalently closed circular plasmid (ccc) due to its superior transfection capability and the absence of single or double strand

breaks which may be detrimental to transgene expression. Even with *in vitro* transcription, a nick in the template within a coding region might result in pre-mature length mRNA products. Such nicked plasmid DNA would often present themselves as open circular forms (oc).

Capillary Gel Electrophoresis is the gold standard here both for analysing chromatography eluates as in-process controls or final DNA product as a quality control method. Orthogonal methods of measuring a single critical quality attribute offer reliability in the context of quality control and aid in vastly improving the quality profile of a product in the context of regulatory acceptability. We employed Anion Exchange High Performance Liquid Chromatography as a second method to characterise plasmid topology initially in purified plasmid DNA before experimenting with samples from various purification stages.

The TSKGel DNA NPR column (Tosoh) in combination with simple Tris-based buffers and a salt gradient elution was very efficient in resolving not only open circular (oc) and covalently closed circular (ccc) conformations but also ccc monomers from ccc dimers.

Our results showed that each individual plasmid required optimized separation conditions for proper resolution of the open circular and supercoiled forms. In the case of an in-stock reporter gene plasmid, we could find a good correlation (2.1 % difference) with the CGE data in measurement of the oc fraction whereby this was dependent upon the total DNA in the injected sample. In the case of a standard backbone plasmid, the percentage distribution of oc and ccc DNA measured by HPLC was more stable over a wide range of total injected DNA in the sample (1.9% and 1.4% CV respectively). By refining the experimental conditions, it would be possible to set up routine parallel measurements by these two different methods, increasing not only the reliability of QC data but also resolving inconclusive observations from any one method.

P371

Fermentation in Single-Use Fermenters for plasmid and *minicircle* DNA production

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There is a surge in demand for GMP-grade plasmid DNA as starting materials for manufacturing crucial viral vectors, especially for late-phase clinical trials and for commercialization. The employment of single-use systems reduces development times through higher batch to batch turnover, allowing quicker supply of clinical material. Single-Use fermenters have been well documented for animal cell culture applications whereas for microbial cultivations, two major limitations were noted: poor oxygen transfer capabilities and insufficient heat removal. The Biostat STR® Microbial single-use fermentation system has been designed specially to overcome these challenges. Following the recommendations for process engineering characterization by the DECHEMA expert group on single-use technology, a volumetric oxygen mass transfer coefficient of up to 675 h^{-1}, a maximum specific power input of 3 kW/m^3 and a maximum cooling rate of 29 $K \cdot h^{-1}$ were noted.

At the R&D facility of PlasmidFactory GmbH, the Biostat STR® Microbial with 40 L working volume was tested for the fermentation of optimal plasmid production strains of *Escherichia coli*. Our tests comprised of batch and fed-batch, small and large plasmids including those for the production of AAV vectors as well as a particularly adapted fermentation process for the

production of *minicircle* DNA. In addition to cascading stirrer speed against the DO level, we set a second level of control through pure oxygen sparging (max. 7 L·min^{-1}) during maximum stirrer speed (500 rpm). Temperature control at a stable 37 °C was successful throughout. Efficient single-use exhaust cooling ensured that the bag pressure never exceeded 10 mbar. A simple batch process yielded a maximum optical density after 16 h fermentation time. We also tested an online biomass density probe that measured transmittance as well as reflection to reliably monitor biomass growth and found correlation with offline data up to OD 14. In terms of biomass yield per batch, DNA product yield per biomass as well as DNA product quality (plasmid DNA homogeneity), the Biostat STR® single-use fermenter offered similar results as compared to conventional stainless-steel fermenters. The purified plasmid DNA from two runs was successfully used for AAV production within HEK293 cells (AAV6 carrying a GFP reporter gene construct). The CGE analysis of DNA from the *minicircle* fermentation showed effective control of induction and the expected level of recombination to yield *minicircle* DNA in the supercoiled conformation. The DNA product after lysis was quantified to be 914 mg from a single batch which is expected to yield over 100 mg of pure *minicircle* DNA. One of the major product quality attributes is the absence of cross-contamination which forms the strongest case for single-use technology. This fermentation system proves that microbial cultivations in the single-use space are no longer a compromise on yield but rather represent a true possibility to shift from conventional sterilizable fermenters. For GMP, this becomes necessary to accelerate production while easing quality control challenges.

P372

Aura+: High Throughput, Low volume Product Stability and Purity Analysis for Gene and Cell Therapies

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Aura+ is the latest instrument designed specifically to detect, count, and characterize subvisible aggregates and extrinsic materials for product quality measurements in both gene and cell therapy applications. Aura+ outputs images, count, size, particle identification and morphological information with 100% sampling efficiency requiring as little as five microliters of sample volume through Backgrounded Membrane Imaging and Fluorescence Membrane Microscopy (FMM). FMM identifies aggregates as cellular, protein, or extrinsic so you can quickly know what is in your sample. Specifically, Aura+ has the capability to detect SYBR® Gold stain, which is used to detect the presence of DNA in AAV aggregation to understand the role of leaky capsids in subvisible particle formation often leading to reduced AAV transduction efficiency and adverse patient responses

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P373

Vial Containment Systems for Gene Therapy Drug Products

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Viral vector-based gene therapy drug products typically are stored and distributed at ultra-low temperature (freezer at -80°C or a container with dry ice at -78°C). There are several requirements of the vial containment system (i.e., vial, elastomer stopper, seal) for these drug products. It must maintain container closure integrity (CCI) performance through fill/finish operations, storage/distribution, and thaw. Also, it must have no adverse interaction with drug product. Finally, it must provide adequate recovery and functionality of vector, whether adeno-associated virus, adenovirus, or lentivirus. These requirements are more challenging than those for a vial containment system for a drug product stored and distributed at room or refrigerated temperature. Understanding performance against these requirements is essential for a manufacturer to decide if a vial containment system is potentially suitable.

The purpose of the present research was a multi-year study to characterize the performance of vial containment systems comprising cyclic olefin polymer (COP) vials, bromobutyl elastomer stoppers [with ethene tetrafluoroethene (ETFE) laminate film facing drug product] and aluminum flip off seals. Characterization was based both on fundamental materials properties and experimental measurements of CCI performance at ultra-low temperatures, reduced potential interaction with drug product, and retention of virus viability. Comparators were vial containment systems comprising glass and polypropylene.

CCI performance over time at -80°C and dry ice storage was evaluated by laser-based headspace analysis with both oxygen and carbon dioxide. Potential interaction with drug product was evaluated by: (a) model proteins exposure to glass and polymer systems and measurement of both particle formation and protein loss, and (b) gas chromatography headspace analysis of stoppers with and without ETFE laminate and measurement of levels of permeated compounds. Virus recovery after freeze-thaw was evaluated by optical density technique (260 nm light) and virus functionality was evaluated by flow cytometry (green fluorescent protein).

Results indicate for CCI Evaluation that was made of systems comprising 2 ml COP vials, 13 mm stoppers, and 13 mm seals that were stored at -80°C for up to two years, no system breach was observed. Very little oxygen ingress was observed over 2 years of storage at -80C and very little CO_2 ingress was observed in case of storage on dry ice for 7 days.

Evaluation of potential interaction with drug product results indicate COP vials, and stoppers under agitated conditions showed low level of particle formation and better protein recovery.

In addition, optimal virus recovery and functionality was observed after storage at -80°C and thaw for selected adeno-associated viruses, adenoviruses, and lentiviruses that were evaluated.

In conclusion, all factors considered (CCI performance, reducing risk of potential interaction, virus viability, materials of construction), vial containment systems comprising COP vials, bromobutyl elastomer stoppers with ETFE laminates, and aluminum flip-off seals, offer very good performance for the ultra-low temperature storage of virus-based gene therapy drug products versus systems comprising glass or polypropylene.

P374

Circulating Haematopoietic Stem and Progenitor Cell show thymus seeding properties and actively contribute to haematopoiesis

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Although most haematopoietic stem/progenitor cells (HSPC) reside in the bone marrow (BM), few circulating HSPC (cHSPC) are also found in the peripheral blood (PB) at steady state. By exploiting deep immunophenotyping, ad hoc designed *in vitro* and *in vivo* assays as well as single-cell RNA sequencing (scRNAseq), we found that healthy donor (HD)-derived cHSPC are phenotypically, transcriptionally and functionally enriched for multi-potent, erythroid and lymphoid progenitors. cHSPC subsets also displayed lower cycling activity and enriched expression of ontologies associated to differentiation, adhesion molecules and immune response/activation, suggesting a pre-activated state of trafficking HSPC at steady state. However, the migratory fate and direction of cHSPC subpopulations as well as their relationship with BM-resident counterpart in humans remain still not fully elucidated.

To gain insight into the migratory properties of cHSPC subsets, we generated a Human Organ-Resident (HuOR) HSPC dataset, by exploiting published scRNAseq datasets from different HD-derived organs. We detected a higher transcriptional similarity between lymphoid-primed cHSPC from our scRNAseq dataset and HuOR thymus-resident HSPC. Moreover, a substantial fraction of lymphoid cHSPC displayed an enriched expression of a gene signature associated with thymus seeding progenitor type 1 (TSP1), a group of low-cycling immature lymphoid progenitors with thymus-emigrant properties. Few TSP1 cells were also detected in lymphoid BM-HSPC subsets, thus suggesting that few thymus-emigrant lymphoid progenitors originate in the BM and preferentially egress into the PB to reach the thymus.

To study circulating vs. resident HSPC differentiation potential and dynamics *in vivo* in humans, we exploited integration site (IS) clonal tracking of cHSPC, BM-HSPC subsets derived from 2 distinct BM sites, and mature PB lineages isolated from 8 patients treated with autologous HSPC-gene therapy (GT) at late follow-ups post-treatment (>2 years), once steady-state hematopoiesis is established. In line with their enriched expression of TSP1 signature, cHSPC shared a higher number of IS with mature T cells with respect to their BM counterpart. Moreover, we found a higher IS sharing between lymphoid BM-HSPC and cHSPC as compared to primitive and myeloid/erythroid BM-HSPC subsets, further supporting that lymphoid progenitors have the highest migratory propensity into the PB.

We observed that IS shared between two BM distant sites are re-captured at higher frequencies in PB with respect to IS not shared between distinct BM sites, thus implying an active role of cHSPC in clonal re-distribution among BM niches. Among all HSPC subsets, primitive HSPC showed the highest sharing with these re-circulating clones, suggesting their higher propensity to

re-enter into the BM. Finally, we found that clones shared between BM- and PB-HSPC display higher haematopoietic multi-lineage output than clones not shared between PB and BM HSPC.

Altogether, our results indicate the key function of steady-state trafficking lymphoid HSPC in the seeding of the thymus, with the aim of locally contributing to lymphopoiesis. Moreover, our IS analysis allowed to unveil the dynamics of HSPC trafficking in humans by supporting their fundamental role in human haematopoietic homeostasis.

P375

Lentiviral vector mediated *in vivo* gene transfer into hematopoietic stem and progenitor cells

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Lentiviral vector (LV) mediated *ex vivo* gene therapy in hematopoietic stem and progenitor cells (HSPC) fulfilled the promise of a long-term treatment for a number of genetic diseases, including primary immunodeficiencies and haematological disorders. However, *ex vivo* manipulation of HSPC and patient conditioning still pose challenges to broad access to HSPC gene therapy. To investigate the feasibility of *in vivo* LV-mediated gene transfer into HSPC, we systemically administered GFP-expressing LV to adult mice or newborns and found only in the latter stable GFP expression in 1% of all blood lineages up to 1-year. We observed comparable GFP marking in hematopoietic organs and in HSPC harvested from the bone marrow (BM), which were able to engraft long-term in busulfan-conditioned mice. LV integration site analysis confirmed common origin of different hematopoietic lineages from multiple clones. These findings indicate successful *in vivo* gene transfer into *bona fide* HSC favoured by unique features of newborn haematopoiesis, such as access to hepatic hematopoietic niches persisting after birth, and HSC-trafficking to the BM. In order to evaluate newborn HSPC potential, we isolated hematopoietic cells from the liver or the BM of newborn mice and transplanted them in conditioned adult recipient mice. We observed similar engraftment and hematopoietic output in mice transplanted with cells isolated from the two sources and this was confirmed even upon secondary transplant. To increase gene transfer efficiency we exploited phagocytosis-shielded CD47-high LV and showed 3-fold higher gene marking in the blood and BM of treated mice. Biodistribution studies showed transgene-positive cells in liver, spleen, lung, kidney, and brain, comprising hematopoietic, endothelial, and liver parenchymal cells. As disease models to test this approach, we selected a primary immunodeficiency caused by adenosine deaminase (ADA) deficiency (ADA-SCID), in which corrected T cells possess a selective advantage over non-corrected cells, and autosomal recessive osteopetrosis (ARO), which is caused in more than 50% of the cases by mutations in the *T cell immune regulator 1* gene (*TCIRG1*) that cause an impairment in osteoclast-mediated bone resorption, impeding proper BM niche formation and HSPC homing to the BM. Firstly, we administered ADA-expressing LV to ADA-SCID newborn mice and showed rescue from lethal phenotype and reconstitution of lymphocyte counts with selective advantage of LV-transduced cells. Twenty weeks after LV administration, we transplanted BM cells from ADA-SCID mice treated as newborns into NSG recipient mice and showed long-term LV marking of around 5% in the BM and lymphoid organs of transplanted mice,

in line with marking observed in donor mice treated as newborns. Finally, we treated newborn osteopetrotic mice with TCIRG1-expressing LV and observed prolonged survival up to 18 weeks of age and remodelling of bone trabeculae. Our work shows *in vivo* gene transfer into HSPC in newborn mice and life-long maintenance of transgene expression, suggesting that special hematopoietic niches and higher HSPC accessibility in this window may be exploited to obtain disease correction, especially when a selective advantage of corrected cells is present. Further studies are ongoing to explore *in vivo* LV gene transfer into HSPC in other disease models.

P376

In vivo induction of -113 A>G HPFH mutation using an adenine base editor results in efficient reactivation of HBF in humanized mouse models of β-hemoglobinopathies

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The recent development of powerful genome editing tools has led to new therapeutic approaches for gene therapy of β-hemoglobinopathies, including the in situ correction of β- globin mutations or the induction of HbF reactivation. Most of these strategies involve nucleases inducing double-strand DNA breaks (DSB), which may cause critical off-target effects and also on-target consequences. Base editors (BEs) in contrast, efficiently install precise nucleotide substitutions without creating DSBs, and thus have emerged as a safer and potentially more efficacious approach over conventional genome editing and for β-hemoglobinopathies in particular, the introduction of HPFH-related mutations using BEs represents an attractive alternative. We have previously shown in vitro that transduction of $CD34^+$ cells from β-thalassemia and SCD patients with a non-integrating, tropic to human HSCs via CD46, HDAd5/35++ vector, expressing a highly efficient adenine base editor (ABE8e) and containing an $mgmt^{P140K}$ gene to allow for enrichment of edited cells after O^6BG/BCNU treatment, mediated efficient -113 A>G conversion and reactivation of γ-globin expression with subsequent phenotypic correction of erythroid cells. The aim of the present study was the in vivo transduction of humanized mouse models of β-thalassemia and SCD, using the HDAd5/35++ ABE8e vector. Xenografts were generated by transplanting NBSGW mice with $CD34^+$ cells from thalassemic or SCD donors to establish human, disease-associated hematopoiesis. Six weeks post transplantation, HSPCs from the chimeric bone marrow (bm) were successfully mobilized to the periphery by G-CSF and Plerixafor. Shortly after the last dose of plerixafor, the mice were intravenously injected with HDAd-ABE allowing selective transduction of the human HSCs. Six days after the in vivo transduction, one course of in vivo selection with O^6BG/BCNU (±) (for expansion of edited HSPCs) was administered, and the mice were sacrificed three months post in vivo transduction. The in vivo transduction with HDAd-ABE8e or/and selection with O6BG-BCNU did not negatively affect human HSCs engraftment reaching up to 60% hCD45+cells in the thalassemic and 23% in SCD humanized

model and leading to human multi-lineage representation in the bm. Increased rates of HBF$^+$cells were observed within the hGlyA$^+$cell population of the chimeric bm post in vivo base editing in Thal (up to 43%) and SCD mice (up to 26.6%), being further increased post selection (up to 63% and 55.4%, respectively). The increased rates of HbF$^+$ cells translated into improved phenotypes (erythrocyte differentiation and maturation) and functional characteristics (reduced sickling, reactivate oxygen species) after erythroid differentiation. The globin protein chains, the in vivo -113 A>G conversion rates and potential off-target editing, are currently being analyzed. Overall, we present a novel approach for effective in vivo gene editing for β-hemoglobinopathies which can potentially overcome several barriers of the current ex vivo gene editing (including leukapheresis and myeloablation) and allow a wider application of and patient access to gene therapy for hemoglobinopathies.

P377

Targeted, safe, and efficient gene delivery to human hematopoietic stem and progenitor cells *in vitro* and *in vivo* using engineered AVID adenovirus vector platform

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Precise editing of human genome using CRISPR-Cas nucleases, base editors, prime editors, or other technologies represents a promising approach for ameliorating or correcting many human genetic diseases, which are caused by mutations in genes that regulate or enable critical developmental, somatic, host defense, or metabolic functions. Although numerous pre-clinical and clinical proof-of-concept studies showed the feasibility of gene correction in desired regions of the human genome in cell cultures *in vitro*, the targeted delivery of gene editing technologies to various specific cell types *in vivo* represents major challenge, limiting utility of gene editing technologies in clinic. Here, we describe the development and analysis of AVIDs, adenovirus-based gene delivery platform, allowing to achieve a highly targeted, safe, and efficient gene delivery to human hematopoietic stem cells *in vitro* and after intravenous administration *in vivo*. The AVID vectors attach to human hematopoietic stem and progenitor cells (HSPCs) via engineered fibers that bind to either CD46 or DSG2 receptors, which are highly expressed on human HSPCs. The efficient entry of AVIDs into these cells is mediated via a mutated penton that was engineered to interact with a6 integrin class, whose expression is restricted to cells with long-term repopulating and stem cell capacity. To reduce off-target virus sequestration in the liver and improve virus safety after intravenous administration, as well as to increase the efficacy of virus production for a cost-effective high-yield vector manufacturing, AVIDs further comprise mutations in hypervariable loops of the major capsid protein hexon. While *in vitro* cell infection with AVIDs leads to about 30% transduction of a pool of human CD34+ cells, the efficacy of transduction of HSPC population, phenotypically defined as CD34$^+$CD38$^-$CD45RA$^-$CD90$^+$ cells ranged from 70% to 99% for individual donors. Single intravenous administration of AVIDs to humanized mice, grafted with human CD34$^+$ cells, after mobilization led to up to 20% transduction of CD34$^+$CD38$^-$CD45RA- HSPCs. Importantly, targeted transduction of a6-integrin-expressing human HSCs phenotypically defined CD34+CD38-CD45RA-CD90-CD49f+ reached up to 19%, with no or only minimal transduction of committed progenitor populations in the bone marrow. Taken together, our study suggests that AVID vectors can be a useful platform for targeted, safe, and efficient therapeutic gene delivery to human HSPCs *in vitro* and in vivo after a single intravenous vector administration.

Conflict of interest statement: DSH and NCDP are co-founders, shareholders, and officers of AdCure Bio, LLC, which develops adenovirus technologies for therapeutic use. DSH and NCDP are listed as inventors on pending and issued US and European patents and patent applications that describe adenovirus technologies. This work was supported by funds from the US NIH grant AI107960 and contracts from AdCure Bio, LLC.

P378

Lentiviral-mediated gene therapy for Diamond-Blackfan anemia: one step closer to the clinic

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Diamond-Blackfan anemia (OMIM #105650) (DBA) is a bone marrow failure (BMF) condition with an estimated prevalence of 5-7 cases per million live births. Hematological clinical signs are predominantly defined by erythroblastopenia, frequently associated with neutropenia and thrombocytopenia. Additionally, 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%). Therefore, as a first step, we focused on the development of an ex vivo gene therapy approach to correct the genetic defect of *RPS19*-deficient cells. To this aim, we generated two different therapeutic LVs with eukaryotic promoters (LVs: *PGK.CoRPS19.Wpre** and *EF1α(s).CoRPS19.Wpre*-LVs*). The proof of concept regarding its efficacy was confirmed by restoration of the expression of RPS19 and the correction of the ribosomal biogenesis defects characteristic of this ribosomopathy using a DBA-like K562 cell model in which *RPS19* was down-regulated with a sh-*RPS19* LV. Subsequently, we focused on the *PGK.CoRPS19.Wpre**-LV to evaluate its efficacy and safety in primary bone marrow HSPCs ($CD34^+$ cells) from RPS19-deficient patients. Transduction of the $CD34^+$ cells from DBA patients with this therapeutic LV significantly increased the number of BFU-E colonies and reverted the red blood cell differentiation defect characteristic of DBA $CD34^+$ cells, as revealed by the increased output of $CD71^+/CD235^+$ mature erythroid cells in vitro. Remarkably, DBA $CD34^+$ cells that had been transduced with the therapeutic LV were capable of repopulating the hematopoiesis of immunodeficient mice and also showed improved erythropoiesis compared to uncorrected samples. In vivo studies also confirmed the healthy status of transplanted immunodeficient recipients, as well as the polyclonal repopulation pattern of corrected hematopoietic cells. Currently, single-cell RNA-seq studies are ongoing to unravel the transcriptomic consequences of gene complementation. Taken together, the preclinical studies conducted in this work strongly suggest that the LV-mediated gene therapy, in particular with the *PGK.CoRPS19.Wpre**-LV, for which the Orphan drug designation “EMA/OD/0000060656” has

already been obtained, should provide an efficient and safe approach to restore the hematological defects characteristic of RPS19-deficient DBA patients.

P379

Long-term follow-up in patients treated with gene therapy for TDT beta-thalassemia and improved transduction towards a Phase IIb clinical trial.

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Transfusion-dependent ß-thalassemia (TDT) is a disorder due to mutations in the gene encoding the ß-globin chain, leading to severe anemia and lifelong transfusion dependence. *Ex vivo* hematopoietic stem cell (HSC) gene therapy has been recently acknowledged as an alternative cure to allogeneic bone marrow (BM) transplantation, with the first product on the US market. In 2015 we started a clinical trial based on autologous mobilized HSC transduced by the GLOBE vector, administered by intra-bone injection, following a reduced myeloablative conditioning in adult and pediatric patients. All patients are alive and well and entered a long term follow up study (NCT03275051). Long-term analysis showed a persistent and stable engraftment of genetically engineered cells in 6 out of 9 patients (transduction effiency, TE, in BM between 28.1 and 80.0%). The clinical outcome in adult patients showed a significant reduction in transfusion requirement. Among the 4 pediatric patients who achieved transfusion independence, Pt 7 and 8, have restarted transfusion at 6 and 5 years of follow-up, respectively. A slow and progressive decrease in VCN and TE has been observed both in peripheral blood and BM cells in Pt8. Differently, Pt 7, carrying severe ß0ß0 mutations, showed a constant marking of transduced cells (around 50%) and restarted transfusion due to need of higher Hb during puberty. Consistently, integration site analysis demonstrated highly polyclonal engraftment but with a reduced number of insertions in patients showing a reduced engraftment of transduced cells with no clonal dominance. These results indicate the need for an *in vivo* threshold of VCN and TE in transduced CD34+ cells to obtain the correction of anemia and associated features. We exploited the use of combined transduction enhancers, in a single hit transduction protocol, to achieve higher VCN/cells and TE reducing culture time, thus preserving the stemness features. Among different enhancers, CSH and PGE2 resulted in higher VCN/cells and TE reaching up to 3-fold increase compared to the previously standard used protocol (STD). We also tested the poloxamer BOOSTA alone or in combination. *In vitro* results showed 2-fold increase in VCN/cells using BOOSTA in combination with CSH or PGE2 as compared to STD. 3.6 fold increase in VCN/cells was reached with triple combination with equal TE. Among protocols, we observed similar growth rate of $CD34^+$ cells with no differences in clonogenic capacity andhematopoietic composition. RNAseq analysis to evaluate the impact of enhancers on primitive subpopulations and pathways is ongoing.To evaluate *in vivo* repopulating capacity of genetically modified cells, we performed biodistribution studies in immunodeficient NBSGW mice. At 16 weeks post transplantation human transplanted cells were able to equally engraft and differentiate in specific lineages in all conditions. In the collected hematopoietic tissues human cells showed VCN comparable to injected cells, indicating persisting marking in NSG-repopulating human progenitors. Overall, these results showed that addition of transduction enhancers to the culture protocol increases VCN/cell and TE in human repopulating cells, allowing to reduce the culture

time preserving long term in vivo marking, and enabling the clinical development of a new Phase IIb trial.

P380

TALEN editing coupled to non-viral DNA delivery enables efficient correction of sickle cell mutation with minimal transcriptional changes and low level of *HBB* KO

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Sickle cell disease (SCD) stems from a single point mutation in the *HBB* gene which results in sickle hemoglobin (Hb). Nuclease-mediated gene therapy approaches provide a relevant therapeutic alternative for patients who are not eligible for an allogeneic stem cell transplantation. We previously reported efficient TALEN-mediated *HBB* gene correction in hematopoietic stem/progenitor cells (HSPCs) leading to therapeutic Hb expression in differentiated red blood cells (RBCs). Furthermore, using ssODN as repair template in HSPCs from healthy donors, mitigated p53 activation with respect to AAV, and resulted in a better maintenance of high-level gene correction *in vivo*.

We further confirmed that using ssODN in SCD patients' HSPCs also led to a lower activation of p53 response compared to AAV, preserving transcriptomic profile and engraftment capacity similar to non-edited HSPCs. Interestingly, TALEN-mediated editing of the unique off-site located in the *HBD* gene led to genomic rearrangements that were negatively selected in the engrafted population *in vivo*.

We also characterized the risk associated to *HBB* gene knock-out by 5' single-cell RNAseq analysis and simultaneously assessed *HBB* genotype and whole transcriptome in edited erythroid cells. We identified <10% erythroid cells harboring bi-allelic indels with a β-thalassemic signature and no major transcriptional changes in corrected compared to mock control cells.

Overall, these data confirmed that the mutant *HBB* gene can be efficiently corrected by TALEN gene editing coupled to ssODN with a low risk of generating β-thalassemic RBCs and validated the use of scRNAseq to comprehensively characterize the impact of gene editing in HSPCs and RBCs.

P381

Hematopoietic Stem Cell Gene Therapy as a Novel Gene Therapy Approach for Severe Crohn's Disease Associated with NOD2-Deficiency

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Deficiency of NOD2 (nucleotide-binding oligomerization domain containing protein 2) demonstrates the strongest genetic association to Crohn's inflammatory bowel disease (CD). Mounting evidence links NOD2 deficiency with poor clinical outcome, particularly in pediatric and early onset CD. CD patients with loss-of-function NOD2 variants, particularly carriers of more than one risk allele, frequently present an aggressive, fistulizing and fibrostenotic disease, requiring multiple surgical resections, and are more frequently refractory to available therapies, thereby highlighting a high unmet need. NOD2 activity within haematopoietic-derived cells regulates gut innate immunity, by sensing its primary ligand bacterial muramyl depeptide (MDP), and thereby invoking immune responses to intestinal pathogens. NOD2-deficiency is implicated in CD pathogenesis through failure to resolve bacterial infections and by loss of tissue homeostasis within the intestinal microenvironment. Here we present preclinical data on OTL-104, an autologous haematopoietic stem cell gene therapy (HSC-GT) which aims to stably restore NOD2 expression in gut resident macrophages, to correct immune dysfunction linked to NOD2-deficient CD pathogenesis.

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 MDP stimulation. 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 (IL-8, TNFa and IL-6). In both NOD2 deficient models, transduction with a lentiviral vector (LVV) expressing NOD2 under the macrophage-restricted chimeric CathepsinG/cFES promoter fully restores NOD2-mediated cytokine responses. Profiling broader cytokine and chemokine responses induced by MDP stimulation in corrected NOD2-deficient patient cells, confirms NOD2 LVV is able to restore an immune profile that is comparable to monocytes derived from $CD34^{+}$ cells from NOD2 wild-type healthy donors.

Equivalent to studies performed using human cell models, myeloid cells derived from murine lineage negative haematopoietic stem/progenitor cells (Lin^{-} HSCs) are also refractory to MDP stimulation, and show an abrogated cytokine response compared to cells derived from WT mice, and can be fully corrected by transduction of Lin^{-} HSCs with NOD2 LVV. Transplantation of Lin^{-} HSCs transduced with the OTL-104 vector in $NOD2^{KO}$ mice was used as an *in vivo* model of gene therapy for CD. Compared to wild-type mice, $NOD2^{KO}$ mice fail to release systemic inflammatory mediators and recruit myeloid cells in response to MDP administration. Transplantation of NOD2 LVV transduced Lin^{-} HSCs restores MDP-induced systemic release of IL-6 and CXCL1, innate mobilization of monocyte/macrophage cells, as well as additional intestinal immune mediators. Transplanted $NOD2^{KO}$ mice display normal haematopoiesis and stable vector copy numbers in haematopoietic cells. Key to the success of our therapeutic approach, histopathological analysis of intestinal *lamina propria* from transplanted mice shows a normal biodistribution and physiological NOD2 gene expression in tissue resident cells. These results

demonstrate the efficacy of OTL-104 in restoring NOD2 expression and function, supporting the therapeutic potential of OTL-104 HSC-GT for long-term correction of NOD2-deficient CD.

P382

Development of an efficient CD34+ cell transduction protocol with the GLOBEAS3 lentiviral vector in the perspective of using umbilical cord blood for the gene therapy of sickle cell disease.

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In sickle cell disease (SCD), pathological sickle hemoglobin (HbS) polymerization leads to red blood cell sickling, responsible for anemia, vaso-occlusion and complications. Different gene therapy approaches have been developed to treat SCD through the transplantation of autologous, genetically-modified hematopoietic stem/progenitor cells (HSPCs). However regardless of the genetic strategy, the efficacy of gene therapy will depend on collecting sufficient numbers of HSPCs and treating patients early, before sequellaes of the disease occur. With the possibility of prenatal or neonatal diagnosis, umbilical cord blood could become a useful source of HSPCs for the gene therapy of SCD in young patients. A clinical protocol DREPACORD was established to collect umbilical cord blood (CB) from neonates with a HbSS SCD genotype at the CHSF hospital (NCT 03876821). Here, we present the results of an optimized transduction protocol of HbS+ CB CD34+ cells with an anti-sickling lentiviral vector (LV). We used the GLOBEAS3 LV which expresses the HbAS3 b-globin mutant containing 3 point-mutations that enhance a-globin interactions and reduce HbS polymerization. In prior preclinical studies, 2 copies of the GLOBEAS3 LV in CD34+ cells from bone marrow of SCD patients provided about 25% HbAS3/HbS ratio resulting in 35% non-sickling erythroid cells (Poletti et al. Molecular Therapy – Methods & Clinical Development, 2018). In our present study, the GLOBEAS3 lentiviral vector was provided by Genethon and was produced in industrial conditions in bioreactors at 200L scale in serum-free conditions. Different transduction enhancers and modalities (vector concentration, number of hits) were tested to optimize the transduction of CB CD34+ cells obtained from the DREPACORD study. Initial set-up experiments conducted with a GFP-LV produced and purified at large-scale showed that 2 consecutive infection cycles with 5 E+07 IG/mL of vector transduced between 65-85% of CD34+ cells depending on the addition of the transduction enhancer cyclosporin H. With the GLOBEAS3 LV using 2 hits of infection in the absence of presence of cyclosporin H, we integrated between 2 and 4 copies of vector in BFU-Es derived from HbSS CD34+ cells, providing between 150-250 % more HbAS3 over HbS as measured by HPLC. No toxicity of cyclosporin was observed on HbSS CD34+ cells. Experiments for in vivo engraftment in immunodeficient mice are ongoing. The data suggest that HbSS CB cells can be transduced with an anti-sickling LV at high levels expected to provide therapeutic effects. Further evaluation of SCD CB cells is therefore warranted to determine their possible use for the gene therapy of SCD.

P383

Exploring the interaction of senescent cells and the immune system in HSPC gene therapy

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Gene therapy (GT) product approval relies on *in vivo* safety studies, based on the knowledge that integration of retroviral vectors nearby cancer genes may result in their deregulation, inducing malignancy. However, these types of studies, other than being costly and time-consuming, may underestimate other adverse events that could occur before or beyond tumorigenesis. Indeed, oncogene (BRAFV600E) activation in human hematopoietic stem and progenitor cells (HSPCs) transplanted into immune-deficient NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice triggers senescence, causing lymphoid impairment, exacerbated secretion of pro-inflammatory cytokines, paracrine senescence, and bone marrow (BM) failure. Thus, oncogene-induced senescence may result in detrimental effects either before cell transformation or independently from it, by impairing immune cells, inducing oligoclonality, and other signs of early aging of the hematopoietic system.

We planned to dissect further the detrimental events occurring in senescence, while also selecting specific senescence biomarkers for the future setup of in vitro safety assays. To identify specific markers related to senescent cells (SCs) in the absence or presence of an active immune system, WT or NSG mice were transplanted with WT mouse (m) HSPCs transduced with lentiviral vectors expressing mBrafV600E or GFP as a control.

In NSG recipients, transplantation of mBrafV600E-expressing cells led to a reduction in peripheral blood cellularity, lymphoid impairment, BM failure, and multi-organ histiocytic infiltration in 92% of animals resulting in a highly penetrant and dose-dependent lethality. On the other hand, only 60% of WT recipients succumbed to BM failure, while the surviving WT mice, unlike NSG recipients, were able to successfully eliminate SCs. At the base of this, NSG recipients exhibited elevated levels of pro-inflammatory cytokines and chemokines, such as CCL-2, -3, -4, -5, IL-2, -6, and -12 in the blood plasma, whereas WT recipients only showed increased CCL-3, -4, and -5. We also performed RNA-seq analysis on sorted myeloid and B cells from both recipients. Enrichment analysis showed upregulation of specific hallmarks in both recipients, such as interferon-gamma and interferon alpha response, inflammatory response, IL-6-JAK-STAT3 response, and IL2-STAT5 response. Moreover, gene ontology analysis of biological processes revealed upregulation of ribosome biogenesis and MHC class II signaling. Regarding known senescence markers, the *Cdkn2a* gene, encoding for the cell cycle inhibitor proteins p16^{Ink4a} and p19^{Arf}, is upregulated in both recipients. Of note, only B cells of WT recipients displayed downregulated cell cycle progression and mitosis-related hallmarks.

With this study, we identified specific signatures related to oncogene-induced senescence that can be further investigated as early genotoxicity biomarkers for cost-effective in vitro assays in future studies. Moreover, our results indicate how the immunological background of recipient mice can influence the elimination of SCs. In the context of HSPC GT treatments, often dealing with immunocompromised patients, the identification of mechanisms for the elimination of SCs will improve the safety of GT strategies. Future experiments will dissect the specific subset of immune cells responsible for the clearance, with the use of recipient mice with different genetic backgrounds.

P384

LT-CRISPR, a novel gene-editing tool for precise editing of the human genome

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Genome editing has emerged as a promising strategy to treat various disorders affecting the hematopoietic system. The importance of precise genome editing lies in the fact that it reduces the likelihood of off-target effects, which can cause unintended changes to the genome and lead to harmful side effects. However, therapeutic applications of DNA nucleases suffers from the intrinsically low frequency of homology-directed repair (HDR) compared to Non-homologous end joining (NHEJ) at target site. Presence or absence of different effectors and homologous template at targeted DNA break directs the DNA repair pathway toward HDR or NHEJ. We have demonstrated that by complementing the necessary effectors with Cas9 we can inhibit NHEJ and increase HDR events at targeted site. Using our previously established HDR-CRISPR, we were able to achieve 3-fold increase in precise genome editing as compared to the unmodified Cas9. Here we developed a novel linked-template CRISPR system (LT-CRISPR) to enrich the repair template at DNA double strand break (DSB). We demonstrate that LT-CRISPR is capable of efficiently capturing a single stranded DNA repair template and deliver it directly to the site of the DSB where it promotes DNA repair with 4 fold higher precision for small amino acid change in comparison to unmodified Cas9. Further screening of effectors involved in DNA repair has led to the identification of new fusion partners for the Cas9 that favour the HDR-mediated resolution of a DSB. Ongoing experiments explore the efficacy of LT-CRISPR in fusion with newly identified effectors to correct Fanconi Anemia (FA) specific mutations in-patient-derived hematopoietic stem cells. We believe LT-CRISPR system offers the opportunity to pursue precise genome editing when DNA repair mechanisms are impaired as a consequence of the underlying genetic defect.

P385

A comparison of different strategies for CRISPR/Cas9-based gene therapies of congenital neutropenia associated with *ELANE* mutations

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Congenital neutropenia (CN) is a bone marrow failure syndrome resulting in low (<500 / µl) peripheral blood neutrophil counts. Besides the risk of severe bacterial infection, patients carry a 15 % risk of developing MDS or AML. CN patients are treated with rhG-CSF chronically. The only curative therapy is a hematopoietic stem cell transplantation with its severe risks, and therefore alternative treatments are urgently needed. Different CRISPR/Cas9-based *ex vivo* gene therapy approaches have been described with their specific advantages and disadvantages. We performed a direct head-to-head comparison of gene editing strategies in hematopoietic stem and progenitor cells (HSPCs) from two *ELANE*-CN patients with different mutations.

We compared the efficacy of the following *ELANE* gene editing strategies: i) universal knockout (uEKO), ii) allele-specific KO (ASKO), iii) HDR-based mutation correction with an AAV6 vector (HDR-AAV), and iv) allele-specific HDR with a ssODN donor (AS-HDR). For the comparison, we used HSPCs carrying p.A57V (CN patient 1) and p.G241V (CN patient 2) *ELANE* mutations. These mutations are in hotspots that lead to unresponsiveness to G-CSF and are associated with a high risk of leukemia transformation. CRISPR/Cas9 was delivered as an RNP by electroporation. Control cells were edited in AAVS1 safe harbor. Gene-edited cells were differentiated to neutrophils. At the end of the differentiation, we assessed the editing efficiency, the differentiation, and reaction oxygen production (ROS).

For CN patient 1, we investigated the uEKO, ASKO, and HDR-AAV. We found 62 % indels in AAVS1, 70 % uEKO, 76 % ASKO, and 10,3 % HDR-AAV with an additional 49.5 % *ELANE* KO. These editing outcomes significantly improved neutrophil maturation from 14.5 % of neutrophils in control edited cells to respectively 57.4 %, 55.2 %, and 45.3 % of neutrophils in *ELANE*-modified groups, respectively.

For CN patient 2 we tested uEKO, HDR-AAV, and AS-HDR. The observed editing efficiencies for CN patient 2 were 86 % indels in AAVS1, 34.4 % uEKO, 80.25 % HDR-AAV, and 0% AS-HDR but 92 % of mutant alleles contained frameshift indels. These editing outcomes significantly corrected the CN phenotype by increasing the portion of mature neutrophils from 13.6 % to 53.7 %, 57.3 %, and 62.8 %, respectively.

The alleviation of CN led to increased ROS production upon fMLP stimulation in both patients, indicating the neutrophils' functionality.

To assess the off-target profile for our guides on the patient's genetic background, we performed GUIDE-Seq in patient-derived iPSC from both patients. For the guides targeting exon 2, we found 10 potential off-targets (OT) for the sgRNA targeting both alleles and 3 OTs for the allele-specific sgRNA. In exon 5 we identified 3 potential OTs for both guides.

In conclusion, all compared approaches restored granulocytic maturation and functionality equally effectively. Unexpectedly, ASKO of CN mutations in late exons rescues neutrophil maturation is unexpected and warrants further investigation. Furthermore, patient-derived iPSCs are an excellent tool for assessing the genotoxicity of gene therapy approaches. Our test revealed a favorable OT profile for the allele-specific sgRNAs. More patients need to be analyzed to confirm this observation.

P386

Recent progress on hematopoietic stem cell gene therapy for adenosine deaminase 2 deficiency

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Loss of function mutations in the adenosine deaminase 2 (ADA2) gene cause an inborn error of immunity known as deficiency of adenosine deaminase 2 (DADA2). Typical manifestations include vasculopathy and immunological and hematological defects, ultimately leading to bone marrow

(BM) aplasia. The current clinical management is a life-long anti-TNF therapy, and hematopoietic stem and progenitor cell (HSPC) transplantation is recommended only in the most severe patients. To assess whether HSPC gene therapy could constitute a valuable treatment to ameliorate HSPC impairment, we developed a third-generation lentiviral vector (LV) expressing a functional ADA2 gene. Transduction of DADA2 patient HSPCs allowed efficient delivery of functional ADA2 in patients' HSPCs cells, as shown by western blot and extracellular ADA2 activity assays. In addition, vector copy numbers (VCNs) in transduced patient HSPCs were similar to those obtained in healthy donor (HD) HSPCs. To assess the clonogenic and multilineage differentiation potential of transduced patients' HSPCs, a colony forming unit (CFU) and an *in vitro* differentiation assay were performed, respectively. CFU assay exhibited high transduction efficiency and VCN among different primitive cell lineages with a concomitant absence of LV-induced toxicity in patients' HSPCs. CFU and *in vitro* differentiation assays did not show any major alterations in the clonogenic and multilineage differentiation potential of transduced DADA2 compared with untransduced patients' HSPCs *in vitro*. Preclinical safety and toxicity studies in immunodeficient mice with ADA2 overexpressing HD HSPCs showed that ADA2 overexpression is well-tolerated and did not impact the *in vivo* multilineage reconstitution potential of HSPCs. Additionally, integration site analysis showed polyclonal engraftment and the absence of genotoxicity. Finally, initial *in vivo* proof-of-concept studies with transduced DADA2 patient HSPC show that these cells can engraft in immunodeficient mice and reconstitute the complete hematopoietic lineage. In conclusion, our results show that LV-mediated ADA2 reconstitution successfully restores ADA2 expression and activity in HSPCs without impacting their clonogenic and differentiation potential. In addition, our preclinical studies in mice show that ADA2 overexpression in HSPCs is safe and well-tolerated without any signs of genotoxicity and that transduced DADA2 patient HSPCs can successfully engraft *in vivo*.

P387

Development of a gene editing approach for the correction of *RPL5*-deficient Diamond-Blackfan anemia hematopoietic stem cells

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Diamond-Blackfan Anemia (OMIM #105650) (DBA) is an inherited bone marrow failure (IBMF) syndrome mainly characterized by red cell aplasia, congenital abnormalities, and increased risk of cancer. The estimated prevalence of DBA is 5-7 cases per million live births. Mutations in more than 24 genes have been associated with DBA, being *RPS19* the most frequently mutated (25%), followed by *RPL5* (11%). A clinical lentiviral-mediated gene therapy approach for the treatment of RPS19-deficient patients is under development in our lab. In the case of RPL5, and due to its direct interaction with MDM2 - the master regulator of p53 - the genetic correction of this gene might require strict endogenous regulation. Therefore, we have developed a universal homologous recombination (HR) gene editing strategy for the treatment of *RPL5*-deficient patients with any mutation type in this gene. Gene editing tools were based on the CRISPR/Cas9 system coupled with adeno-associated viral vectors serotype 6 (AAV6) harboring a codon-optimized sequence of *RPL5*-cDNA (*CoRPL5*). Inefficient HDR-mediated gene editing was initially observed in bone marrow (BM) $CD34^+$ cells from healthy donors (HD) when single-stranded AAVs (ssAAV) were

used. Therefore, further optimization was required using healthy donor BM and cord blood (CB) $CD34^+$ cells and single-stranded (ssAAV6) and self-complementary (scAAV6) AAV6 vectors at different multiplicities of infection (MOIs). The scAAV yielded a higher efficiency and lower toxicity than the ssAAV, even when used at MOIs 10-30 times lower. In CB-$CD34^+$ cells, HR analysis of the scAAV showed a mean value of 79% edited alleles detected by ddPCR and 73% of edited colonies (CFCs) at an MOI of $3x10^3$ genome-copies per cell (GC/cell). In BM-$CD34^+$ cells, ssAAV were too toxic, but the use of scAAVs allowed to achieve a mean of 50% edited alleles detected by ddPCR at MOI of $3x10^2$ GC/cell with minimal toxicity. Experiments to test gene editing efficiency after *in vivo* transplant in NBSGW mice are currently ongoing.

P388

Novel adenovirus vectors for efficient transduction of human hematopoietic stem cells and peripheral blood monocyte subsets

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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 (Ad) type 5 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 Ads (Ad3, Ad7, Ad11, Ad14, Ad16, Ad21, Ad34, Ad35, Ad50), three species D Ads (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. Then the most efficient candidates were converted into first generation vectors (fgAds) expressing GFP reporter gene for further transduction study.

We found that Ad7, Ad11, Ad16, Ad34 and Ad35 show robust cellular entry efficiencies, evidenced by qPCR detection of viral genome and flow cytometry analysis of adenovirus hexon protein at 3- and 6 hours post infection. Next, we converted these wild-type candidate viruses into E1-deleted fgAds expressing GFP reporter gene. The HSPC transduction with GFP-expressing fgAds was analyzed via flow cytometry 48 hours post infection. All these novel vectors showed efficient HSPC transduction. In addition, to explore blood cells that can be relevant for in vivo gene therapy, we transduced peripheral blood mononuclear cells from healthy donors with these vectors and found that in contrast to Ad5, vectors based on Ad11, Ad16 and Ad35 show higher transduction efficiencies in monocytes, NK- and T-cells. Moreover, we performed the transduction study on cancer cell lines originating from hepatocytes. In both Huh7 and HepG2 cells, we observed significantly reduced transduction from these species B adenoviruses compared to Ad5. Furthermore, since the helper-dependent adenovirus system is

the best choice for direct in vivo delivery, some promising adenovirus types will be converted to helper viruses to enable the generation of helper-dependent adenoviruses.

This study identified a few other species B human adenoviruses with efficient HSPC transduction. The newly generated vectors hold high potential for in vivo HSPC gene therapy as well as direct transduction of blood lineage subsets.

P389

Mining the genome for erythroid specific enhancers to optimize gene therapy vectors for beta hemoglobinopathies.

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Gene therapy represents a revolutionary arm of modern medicine since it has already enabled curative single dose treatments for several diseases, with or without a genetic etiology. Spatiotemporal regulation of the gene therapy effect to the affected tissue, can highly reduce unwanted side effects caused by ectopic expression and increase both safety and efficacy of the method. In the current study we developed a direct genome screening-to-application pipeline to optimize the design of clinically applicable gene therapy vectors. As a proof of concept, we focused on improving gene therapy strategies for beta hemoglobinopathies. Not overlooking the promising results that emerged from lentiviral mediated gene therapy using autologous hematopoietic stem cells (HSCs), limitations still exist. The incorporation of the long (~3kb) “micro” β-globin LCR, to enhance expression of the transgene results in low viral titers and reduced transducibility of HSCs, thus negatively affecting efficiency and increasing the overall therapy cost. Identifying novel regulatory elements residing beyond the beta globin locus to drive cell-type and stage specific expression is a key challenge that has not yet been explored in the context of HSC gene therapy. Capitalizing on our high-resolution temporal atlas of developmentally responsive DNaseI hypersensitive sites during human adult ex vivo erythropoiesis, we interrogated the enhancer ability of 15.000 short (200bp) genomic sequences. These elements display erythroid-specific activity, high maturation-stage specificity and a wide range of enhancement activity that enables the precise tuning of transgene expression levels during erythroid differentiation. At least one of these novel enhancers (NE), incorporated in a BCL11A targeting short-hairpin RNA (BCL11A-shRNA) vector was able to successfully challenge the canonical beta globin μLCR enhancer which has been developed and perfected for almost 30 years. The new therapeutic vectors yielded a 2-5 fold increase in viral titers compared to the μLCR respective vector with similar therapeutic gene expression in an erythroid cell line. Within the transduced populations, γ-globin expression was equally increased in all groups compared to untransduced cells ($p<0.004$). Importantly, the new vectors achieved a significantly higher γ-globin expression compared to the μLCR vector in vivo, in NBSGW mice post xenotransplantation ($p=0,007$). Transduction of CD34+ cells derived from thalassemic patients with the shRNA

vectors, yielded a significant increase of %HbF+ cells within the enucleated cell populations, with the difference being higher in the case of the NE transduced cells (p=0.009 NE-shRNA, p=0.02 µLCR-shRNA vs untransduced cells). Additionally, an almost complete restoration of the imbalance between alpha and beta-like globin chains was observed (beta-like/a-globin: 0.94±0.03 NE-shRNA, 0.92±0.02 µLCR-shRNA vs 0.76±0.07 untransduced, p=0.009 NE-shRNA, p=0.02 µLCR-shRNA). This led to a significant improvement of the thalassemic cell phenotype, in terms of proliferation, maturation, oxidative stress and morphology. Collectively, our data provide evidence that the replacement of µLCR by a novel, compact erythroid enhancer, is feasible and successful, surpassing current state-of-the-art gene therapy vector designs.

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Understanding the regulatory functions of ADA2 during inflammation and haematopoiesis to enhance gene therapy efficacy

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Deficiency of adenosine deaminase 2 (DADA2) is a recently defined inborn error of immunity caused by loss-of-function mutations in the adenosine deaminase 2 (ADA2) gene. Clinical manifestations of DADA2 include vasculopathy and immunological and haematological abnormalities, ultimately resulting in bone marrow (BM) failure. In addition, DADA2 patients' macrophages show an increased inflammatory profile. A major gap exists in our knowledge of the regulatory functions of ADA2 during inflammation and haematopoiesis, mainly due to the lack of an ADA2 orthologue gene in rodents. Understanding these mechanisms is essential for developing new precise treatments, including gene therapy. We performed an in-depth phenotypic analysis of the BM of DADA2 patients. In the BM, DADA2 patients exhibited a significant reduction of HSPCs, myeloid and lymphoid cells compared to age-matched HDs. BM HSPCs also showed an impaired clonogenic and multilineage differentiation potential. Similarly, the number of circulating HSPCs and myeloid cells in the PB was also reduced. In correspondence to the phenotype in DADA2 patients, ADA2 KD in zebrafish caused a significant decrease in HSPC numbers and increased number of inflammatory cells, which was partially corrected by administration of recombinant human ADA2. To investigate the DADA2 defects in the hematopoietic niche, we focused on the mesenchymal stromal cells (MSCs) that regulate HSPC homeostasis. We found an impaired clonogenic capacity and reduced proliferation of DADA2 MSCs, leading to premature cell exhaustion in vitro (p3-4). DADA2 MSCs expressed low levels of hematopoietic supportive genes, while senescence markers increased compared with HD's MSCs. This indicates an altered function of DADA2 MSCs, which may contribute to HSPC reduction in the BM. In conclusion, our data reveal a role of ADA2 on the HSPC compartment and haematopoiesis, which also impacts the stromal BM niche.

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Starting a knock-in-based gene editing for Congenital Dyserythropoietic Anemia type II in hematopoietic stem and progenitor cells

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Congenital dyserythropoietic anemias (CDAs) are a group of inherited anemias that affect the development of the erythroid lineage. CDA type II (CDAII) accounts for around 60% of all CDA cases. CDAII patients suffer from ineffective erythropoiesis, morphologic abnormalities in erythroblast within the bone marrow, hemolysis, and iron overload. CDAII is caused by mutations in the *SEC23B* gene, with more than 100 hundred pathological variants described throughout the gene. Allogeneic hematopoietic stem cell transplant (HSCT) represents the only curative option for this disease but implies serious side effects. Autologous HSCT of genetically corrected cells would mean a definitive treatment for CDAII.

To address the correction of most CDAII patients, we have established a gene editing approach to correct SEC23B deficiency in hematopoietic stem and progenitor cells (HSPCs) based on knock-in wild-type *SEC23B* cDNA at the endogenous locus, following a straightforward process. First, we designed and tested different sgRNAs targeting the initial sequence of the *SEC23B* locus in human HSPCs. Then, we selected the most active sgRNAs and performed GUIDE-Seq analysis to assess their off-target potential, and the sgRNA with the lowest potential off-target frequency was chosen. Based on the target sequence of this sgRNA, we designed two rAAV-based knock-in donors to insert either a wild-type *SEC23B* cDNA or a turbo GFP (tGFP) cDNA, together with the bovine growth hormone polyadenylation (bGH polyA). Both cDNAs would be expressed under the control of the *SEC23B* promoter. Next, we nucleofected healthy HSPCs cells with Cas9/sgRNA ribonucleoproteins and transduced with the rAAV knock-in donors, and we detected up to 35% edited cells with wild-type *SEC23B* cDNA and up to 80% HSPCs expressing tGFP. Additionally, we confirmed the expression of wild-type *SEC23B*-bGH-polyA mRNA in HSPCs gene-edited with the wild-type SEC23B donor by RT-PCR assay. This result showed the functional control of the exogenous *SEC23B* cDNA expression by the endogenous SEC23B promoter. Furthermore, to evaluate the potential correction of SEC23B deficiency, we set up a combination of *SEC23B* knock-out together with tGFP cDNA knock-in in human HSPCs and their subsequent *in vitro* erythroid differentiation. We got an efficient tGFP knock-in percentage of erythroid cells derived from $SEC23B^{KO}$ HSPCs over the *in vitro* erythroid differentiation.

In summary, we have established a knock-in gene editing approach for SEC23B deficiency, which might be used as potential gene therapy for CDAII.

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Successful reactivation of gamma Globin gene expression using designer epigenome modifiers offers an innovative therapeutic option for β-Hemoglobinopathies

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β-hemoglobinopathies are severe genetic disorders caused by mutations affecting the production of the β-globin chain. The clinical severity of these pathologies can be effectively mitigated by the reactivation of γ-globin (*HBG*) genes, resulting in elevated production of fetal hemoglobin. In this study, we aimed to investigate the epigenetic landscape of the γ-globin promoter and utilize designer epigenetic modifiers (DEMs) to activate the *HBG* (γ-globin) gene through precise epigenome editing. We first identified the nature of epigenetic marks present at the *HBG1/2* promoters of HUDEP-2 cells that recapitulate adult erythroid cells by expressing primarily b-globin and having the gene coding for g-globin inactive. We confirmed the presence of epigenetic marks indicative of gene inactivation at the *HBG1/2* promoters, such as enriched H3K9me3 and H3K27me3 and high DNA methylation, via ChIP-qPCR and bisulfite sequencing, respectively. We then developed activating DEMs targeting three different positions within the proximal γ-globin promoter and tested their ability to reactivate *HBG* expression in HEK293T cells that typically do not express γ-globin. We delivered DEM to the cells in form of *in vitro* transcribed mRNA via lipofection. Two days later, we evaluated *HBG* expression levels using quantitative polymerase chain reaction (qPCR). We show that the three DEMs were highly efficient in reactivating *HBG* expression with the most effective exhibiting a remarkable 43-fold increase in γ-globin levels. Furthermore, we evaluated the activity of these DEMs in clinically relevant hematopoietic stem and progenitor cells (HSPCs), reaching up to 13-fold increase in γ-globin gene expression, two-days after transfection. Further experiments are ongoing to dissect the long-term effects of epigenome editing on *HBG* expression. The promising outcomes achieved thus far highlight the excellent potential of designer epigenome modifiers in targeting the *HBG* promoter to reactivate γ-globin expression. This approach holds great potential as a novel strategy for the treatment of β-Hemoglobinopathies and paves the way for a new era of therapeutics.

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Proof-of-concept for a one-step gene therapy for STAT1 gain-of-function

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Inborn errors of immunity (IEIs) can lead to increased susceptibility to pathogens and are often associated with severe non-infectious comorbidities. The monogenic nature and confinement to

the hematopoietic system of several IEIs would allow curation through gene therapy. However, most efforts focused on IEI where gene addition can be applied, merely disregarding gain-of-function (GOF) disorders. Signal transducer and activator of transcription 1 (STAT1) is a pivotal transcription factor in the immune response. Autosomal dominant (AD) GOF mutations can cause a triad of chronic mucocutaneous candidiasis (CMC), bacterial infections and autoimmunity, although also distinct clinical manifestations, such as isolated lymphoproliferation, have been described. More than 100 different missense STAT1 GOF mutations have been described. Currently, allogeneic hematopoietic stem cell transplantation is the only curative option, but comes with high morbidity and mortality. Here, we provide a proof-of-concept for a gene knock-in strategy in the STAT1 genomic locus by employing engineered virus-like particles and recombinant adeno-associated viral vectors to deliver CRISPR/Cas9 components and a donor template in cultured HEK293T and THP-1 cells. Exploiting the homology-directed repair mechanism after Cas9-induced double-strand break, we report targeted integration of a codon-optimised STAT1 cDNA cassette in the STAT1 locus and achieve the endogenous regulation by the STAT1 promoter. Currently, we are assessing the rescue of the phenotype. In a next step, we plan to apply our approach in patient-derived cells to corroborate our results and rescue of the phenotype ex vivo. In conclusion, our study highlights the potential of a CRISPR/Cas9-mediated gene therapy for patients holding any GOF mutation causing STAT1 GOF.

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Interferon-gamma-primed mesenchymal stem cells promote the engraftment of human cord blood hematopoietic stem cells in NOD Scid Gamma mice

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Background and aims: Hematopoietic stem cell transplantation (HSCT) is a curative option for a variety of hematologic diseases. Umbilical cord blood-derived HSCs (CB-HSCs) transplantation has shown relatively poor engraftment compared with other sources due to the limited cell number contained in a cord blood unit. Studies have shown that co-transplantation of mesenchymal stem cells (MSCs) with HSCs reinforces the engraftment of HSCs. This study aimed to investigate whether interferon-gamma (IFN-γ)-primed MSCs could further enhance the engraftment of human CB-HSCs than naïve MSCs.

Methods: MSCs derived from Wharton jelly with or without IFN-γ-priming were analyzed by single RNA-sequencing. Next, we performed transplantation of 1 x 10^4 CB-CD34+ cells (Group A), CD34+ cells plus naïve MSCs (Group B), and CD34+ cells plus IFN-γ-primed MSCs (Group C) in Busulfan-conditioned NSG mice. At 6 weeks post-transplant, the engraftment rates were compared among groups by measuring the percentage of human CD45+ cells in bone marrow by flow cytometry analysis.

Results: Single RNA sequencing revealed that 729 genes were upregulated and 477 genes were downregulated in IFN-γ- primed-MSCs. Gene Ontology (GO) analysis demonstrated that most of those changes are related to external stimuli, defense responses and protein bindings. The median percentage of human $CD45^+$ cells in bone marrow of Groups A to C was not statistically different

(3.54%, 3.61%, and 4.49%, respectively; P=NS). The highest engraftment rate achieved in each group was 7.58% (Group A), 13.20% (Group B), and 20.80% (Group C), respectively.

Conclusion: This study showed the gene expression patterns of MSCs change upon IFN-γ-priming. With a limited number of CD34+ cells, co-transplantation of IFN-γ-primed MSCs tended to promote engraftment better than naïve MSCs. Additional studies are needed to verify the engraftment promoting effect of IFN-γ-primed MSCs by further limiting CD34+ cell dose and optimizing conditioning for engraftment tests.

Our findings suggest that co-transplantation of IFN-γ-primed MSCs with CB could be a novel and promising approach to overcome poor graft performance following CB transplantation.

P395

Transcriptomics analysis unveils alterations on the hematopoietic support capacity of bone marrow mesenchymal stromal cells manufactured using human platelet lysate (hPL) - *versus* fetal bovine serum (FBS) supplementation

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Cord blood presents several advantages over other sources of hematopoietic stem and progenitor cells (HSPC) for transplantation, namely lower alloreactivity and easier collection. Nevertheless, due to low HSPC content, *ex vivo* expansion is necessary before transplantation in adults. Since mesenchymal stromal cells (MSC) are a major constituent of the hematopoietic niche and regulate HSPC *in vivo*, MSC-HSPC co-culture systems have been clinically explored. Despite the advantages of an MSC-based co-culture, incorporating a new cell type adds complexity to the manufacturing of cell-based products. Nowadays, it is essential to maintain xeno(geneic)-free conditions during the entire production pipeline. Human platelet lysate (hPL) is a xeno-free alternative to fetal bovine serum (FBS), which has been the standard supplement for MSC expansion. hPL preserves main MSC identity characteristics and has been extensively used in pre-clinical and clinical research. Nevertheless, since minor changes to culture conditions can affect MSC functionality, we thoroughly explored the impact of hPL supplementation on MSC capacity to support HSPC expansion.

We used three MSC donors from bone marrow isolated with hPL- or FBS-supplemented medium. MSC were expanded to reach three conditions: (1) continuously expanded in the isolation culture medium (Direct); (2) adapted to a different medium (Adapted); (3) adapted to a different medium and then re-adapted to the original one (Re-adapted). Co-culture systems for HSPC expansion using the abovementioned conditions were established. Bulk transcriptomics of MSC was performed to identify differences in gene expression between MSC expanded with FBS (FBS-MSC) and hPL (hPL-MSC) that could affect their hematopoietic support capacity.

The culture medium used to expand MSC affected their properties. As expected, hPL-MSC presented higher proliferation capacity and slightly different morphology. Interestingly, transcriptomic data showed two clear clusters for hPL- and FBS-MSC, regardless of the adaptation regimen or donor. 13% of the genes were differentially expressed (DEGs) between Direct hPL and Direct FBS, affecting mainly extracellular matrix organization/composition, taxis, and cell signalling. Only 1-2% of genes were DEGs between Direct and Adapted conditions of the same medium, suggesting that the transcriptome of MSC is similar whether cells are isolated from or adapted to a particular medium. Concerning MSC hematopoietic support capacity, hPL-MSC feeder layers diminished HSPC proliferation capacity. After a 7-day expansion, approximately 2.4 less $CD34^+$ cells were obtained when using hPL-MSC instead of FBS-MSC. Top-down and bottom-up approaches suggest that several pathways affected MSC capacity to support HSPC expansion, including cell-cell signalling mechanisms and TGF-beta, PI3K-Akt, or Wnt signalling pathways. hPL-MSC showed higher expression of genes that inhibit HSPC proliferation (eg: SPP1,MMP14,BMP4,DKK2,EBF2,WNT5B/7B/9A) and lower expression of genes that promote it (eg: JAG1,BMP6,DCN,CDH3/15,ALCAM,JAM2,ITGA1/8/9,IL6,LEPR,WNT2B,FGF7,IGF1/2,IGFBP1/2/4). These factors affect the Notch, TGF-beta, JAK/STAT and Wnt signalling pathways, and cell-to-cell adhesion mechanisms on HSPC, all of them known to greatly regulate HSPC proliferation and differentiation.

Changing the culture medium used for MSC manufacturing greatly affected MSC properties, highlighting the importance of understanding the effects that translational changes may have on cell function. Furthermore, identifying specific genes responsible for MSC's hematopoietic support capacity opens the possibility to optimize co-culture systems and restore MSC properties under xeno-free conditions.

P396

Automated GMP-compatible CRISPR-Cas9 editing of hematopoietic stem and progenitor cells (HSPCs) for the treatment of ß-hemoglobinopathies

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ß-hemoglobinopathies are a group of monogenic disorders that are caused by mutations in the ß-globin gene, leading to transfusion-dependent anemia and multi-organ failure in patients. The only effective treatment option is HLA-compatible hematopoietic stem cell transplantation which is particularly limited by donor availability. Therefore, gene therapy approaches hold enormous potential for the cure of such diseases and have emerged in the past years. One of the gene-editing strategies is based on generating knock-downs of *BCL11A*, a gene involved in the negative regulation of fetal hemoglobin (HbF), to promote the resurgence of HbF expression. This approach was previously demonstrated in preclinical studies using the CRISPR-Cas9 technology and needs to be processed by good manufacturing practice to ensure the quality and safety of therapeutic products for patients. In this study, we demonstrate the automated GMP-compatible CRISPR-Cas9 editing of hematopoietic stem and progenitor cells (HSPCs) for the treatment of ß-hemoglobinopathies, using the CliniMACS Prodigy® platform including the CliniMACS Electroporator (Miltenyi Biotec). We successfully simulated a large-scale clinical scenario, yielding

100 million HSPCs with high editing efficiency. *In vitro* erythroid differentiation and high-performance liquid chromatography analyses corroborated fetal hemoglobin resurgence in edited samples, supporting the feasibility of running the complete process of HSPC gene editing in an automated closed system.

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Optimized Artificial miRNAs Delivered by AAV9 Dramatically Improve Survival, Respiratory and Motor Functions of SOD1^{G93A}-ALS Mice

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Autosomal dominant mutations in superoxide dismutase 1 (*SOD1*) gene cause motor neuron degeneration and are linked to 10-20% of familial- and ~2% of sporadic Amyotrophic lateral sclerosis (ALS), a fatal disease for which an effective treatment is urgently need. Gene silencing of SOD1 using ASO or siRNA has shown modest clinical benefits but require repeated dosing. Here we use AAV9 to deliver optimized artificial microRNAs (amiRs) targeting SOD1 to treat ALS-SOD1 through single dosing. We chose to use an miR-33-based amiR scaffold in our SOD1-amiR design because our previous studies showed that this scaffold results in more effective gene silencing, less off-target activity, and fewer truncated AAV9 genomes compared to conventional shRNAs and other scaffolds.

We further tested three combinations of promoters and scaffolds: 1) an amiR embedded in the mouse miR-33 scaffold driven by the cytomegalovirus enhancer/chicken β-actin promoter (CMVen/CB-amiR); 2) an amiR embedded in the human miR-33 scaffold driven by a promoter derived from the endogenous human survival motor neuron 1 (hSMN1-amiR) promoter; and 3) the above two amiRs driven by the hSMN1 promoter (hSMN1-dual-amiR).

These AAV9-amiR vectors were intravenously injected into three cohorts of SOD1^{G93A} mice (60-68 days of age) at a dose of 1.0 x 10^{14} vg/kg. PBS-treated transgenic animals and the age-matched non-transgenic litter mates were used as controls. Compared to a median survival of 117 days (n = 17) observed in PBS-injected SOD1^{G93A}mice, the median survival of mice treated with the CMVen/CB-amiR vector (n = 5) was extended by 21 days to 138 days. Strikingly, the median survival of the hSMN1-amiR (n = 16) and hSMN1-dual-amiR (n = 17) treatment groups were extended up by 35 days and 94 days, respectively. To gauge diaphragm function and breathing, ventilation was quantified using whole-body plethysmography on day 105. When compared to age-matched litter mates, PBS-treated SOD1^{G93A} mice showed hypercapnia in minute ventilation, peak expiration and inspiratory flows. In contrast, hSMN1-dual-amiR vector-treated SOD1^{G93A} mice performed at the same level as the age-matched litter mates. Remarkably, neither rotarod nor grip tests showed any significant difference between the hSMN1-dual-amiR-treated group and age-matched litter mates on day 190. *SOD1* levels measured on day 105 by qRT-PCR showed a remarkable reduction in the brain stem (down by 46 ± 12%; p< 0.0001) and the spinal cord (down by 35-45%; P< 0.0001) in SOD1^{G93A} mice treated with AAV9-hSMN1-dual-amiR, compared to PBS-treated controls. *SOD1* levels in peripheral tissues such as liver, heart, quadriceps, and diaphragm from same animals were also dramatically reduced. Thus, a single intravascular injection of AAV9-hSMN1-dual-amiR vector in 60-68 days old SOD1^{G93A} mice significantly reduces SOD1 expression within CNS and non-CNS tissues, efficiently preserves respiratory and motor

functions, and extends the survival by 94 days which has not been reported for this SOD1-G93A mice. These results suggest that a clinical evaluation of this strategy is warranted.

P400

Allele-specific silencing of mutant ATXN3 rescues motor deficits and neuropathology in a severely impaired mouse model of spinocerebellar ataxia type 3 (SCA3) upon intra-cisterna magna administration

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Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease, is an autosomal dominantly-inherited and fatal neurodegenerative disorder characterized by an over-repetition of the CAG trinucleotides located in the *ATXN3* gene. This abnormal expansion leads to a toxic gain-of-function of the ATXN3 protein, which results in neuropathology and neurodegeneration in various brain regions, particularly in the cerebellum. Currently, no therapy is available to modify disease progression. Thus, we investigated the therapeutic potential of an AAV encoding artificial microRNAs (miATXN3) directly targeted to the mutant allele, upon intra-cisterna magna (ICM) administration in severely impaired transgenic SCA3 mice. Analysis of motor performance revealed significant and robust improvements in coordination, balance, and gait at 5-, 8- and 11-weeks post injection (assessed by rotarod performance, beam-walking testing and catwalk XT analysis). Importantly, amelioration of neuropathology was also observed through histological analysis, with reduction in the number of ATXN3 inclusions and prevention of layer thickness shrinkage within the cerebellar lobules of AAV9-miATXN3 injected mice, when compared with vehicle-treated mice. These results were corroborated by the observed dose-dependent reduction of mutant ATXN3 mRNA levels in the cerebellum. Furthermore, widespread detection of AAV genome copies and miATXN3 levels in disease-relevant brain regions was observed at the end of the experiment (13 weeks post-ICM injection). Overall, our results support an AAV-based therapy for SCA3 and constitute a step towards closer clinical translation for SCA3 patients.

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Single cell analysis of the glioblastoma microenvironment in patients transplanted with hematopoietic stem cells engineered with a tumor-selective interferon-alpha cassette

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Glioblastoma multiforme (GBM) is a highly malignant, fatal human brain tumor characterized by low T cell infiltration and a strongly immunosuppressive myeloid tumor microenvironment (TME). We are conducting a phase 1/2a study in newly diagnosed patients presenting with (u)MGMT GBM (NCT03866109) evaluating safety and biological activity of Temferon, a hematopoietic stem cell (HSC)-based gene therapy acting on the myeloid GBM TME. We hypothesized that stably engrafted, genetically-engineered HSC produce myeloid cell progeny that home to the tumor and integrate into the TME, where they selectively release alpha-interferon (IFN-a) and stimulate anti-tumor immunity. As of April 30th, 2023, 19 uMGMT GBM patients in 4 cohorts received incremental doses of Temferon obtaining long-term engraftment of IFNa-transduced HSC without dose-limiting toxicities. We performed scRNAseq and scTCRseq analysis of second surgery GBM tumors from n=5 patients post Temferon gene therapy and n=6 control patients treated according to best standard-of-care. Findings were validated on an extended control group encompassing >100 GBM patients from publicly available scRNAseq datasets. Unsupervised clustering of the myeloid TME defined 15 distinct cell types including monocytes, dendritic cells, microglia and multiple macrophage states that cover the M1 to M2 spectrum. Patients from the Temferon group showed an increase in pro-inflammatory (M1-like) and a decrease in hypoxic (M2-like) macrophages. Of note, this population shift was particularly evident when comparing a stable with a progressive tumor lesion biopsied contemporaneously in one GBM patient, reproducing preclinical findings from a GBM mouse model (PMID: 35857642). Analysis of the T cell compartment highlighted an overall increase of $CD8^+$ T cells (mainly effector T cell subsets) and a decrease in $CD4^+$ T cells in Temferon patients. By mapping published signatures of antitumor neoantigen-reactive T cells (NeoTCR; PMID: 35113651) onto the GBM T cell landscape and integrating this data with clonal frequency metrics from concurrent scTCRseq data, we observed an approximately 3-fold increase of predicted tumor-reactive $CD8^+$ T cells bearing expanded clonotypes (>1% within each patient) in Temferon vs. control patients. Intra-population differential gene expression analysis, followed by Gene Set Enrichment Analysis showed strong up-regulation of IFN-a and inflammatory responses in most of the clusters from myeloid and T cells, but also CD45- tumor and stromal cell compartments from Temferon patients suggesting pervasive IFN-a payload delivery into the GBM TME. These data indicate recruitment and integration of Temferon progeny into the TME, where they locally release IFN-a and reprogram the myeloid and lymphoid TME.

P402

Long-term efficacy of AAV-*Npc2* gene therapy for severe Niemann-Pick Type C2 disease

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Niemann-Pick type C2 (NPC2) is an ultra-rare lysosomal storage disease inherited in an autosomal recessive manner. This disease is caused by mutations in *NPC2* gene, which produces a protein involved in cholesterol trafficking from the endo-lysosomal compartment. Patients develop severe, progressive neurodegenerative disorder with relatively mild peripheral pathology. Affected patients show premature death. Currently, there is no effective treatment for NPC2 patients, thus representing a highly unmet medical need. Here, we first developed a new mouse model of NPC2 disease that exhibited the main severe pathological alterations detected in NPC2 patients. Next, we assessed whether intra-cerebrospinal fluid (intra-CSF) administration of AAV9-*Npc2* vectors to NPC2 mice, with already established severe pathology, may offer lifetime treatment. In contrast to non-treated NPC2 mice that all were death by 3 months of age, AAV9-mediated *Npc2* gene transfer resulted in long-term increased *NPC2* expression in CNS, leading to correction of unesterified cholesterol storage and lysosomal pathology in CNS, increased myelination, and reduced neurodegeneration (Purkinje cells) and neuroinflammation for over 8 months of age. After AAV9-*Npc2* delivery, liver was also efficiently transduced, providing a long-lasting, peripheral source of NCP2 protein, which allowed to correct cholesterol accumulation and lysosomal pathology in peripheral organs of 60-day- and 8-month-old NPC2 mice. Furthermore, AAV9-*Npc2* treatment also resulted in normalisation of locomotor deficits at short and long-term, improved body weight and considerably prolonged survival up to one year of age. Altogether, our data demonstrated that a single intra-CSF administration of AAV9-*Npc2* vectors may represent an effective gene therapy to treat even the severe phenotype of NPC2 disease and set the bases for the future translation into clinic.

P403

AAV9-mediated gene replacement therapy for Spastic Paraplegia 47

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Mutations in any one of the four subunits (ε, β4, μ4 and σ4) comprising the AP-4 complex results in a form of Hereditary Spastic Paraplegia (HSP), often termed AP-4 deficiency syndrome. This deficit of AP4 function disrupts neuronal intracellular trafficking, resulting in severe intellectual disability and progressive spasticity of the lower limbs of patients. Through a gene therapy approach we aim to replace the mutated subunit and restore some degree of function for these patients, in particular we look at AP4B1 subunit deficiency, known as SPG47. Our in vitro studies on patient's fibroblast and Ap4b1 deficient neurons show restoration of AP4 complex and ATG9A

(a known cargo of the AP-4 complex, that is upregulated and mislocalised in the trans-golgi network through AP4 disfunction). We have established a CRISPR-mediated Ap4b1-knockout mouse model (Ap4b1-/-) presenting the neuropathological hallmarks of AP-4 deficiency syndrome, this includes corpus callosum thinning, lateral ventricle enlargement, striking mislocalisation of ATG9A, motor co- ordination deficits, , and a hindlimb clasping phenotype associated with neurodegeneration. Cisterna magna delivery of AAV9-mediated expression of AP4B1 in Ap4b1-/- in juvenile and adult mice has shown strong evidence for a substantial rescue of the detectable pathological hallmarks. Biochemical and behavioural assessment for this AP4B1 gene replacement therapy revealed promising results including reducing ventricle enlargement and corpus callosum thinning observed in SPG47 mice. This study has provided us with a platform for progressing this approach towards clinical development where long-term preclinical rodent and NHP regulatory safety studies have already shown no adverse effects of the treatment. Our approach is progressing towards IND in coming months.

P404

Preserving Neurons by controlling inflammation: The Role of IL-10 in Modulating the Immune Response in Parkinson's Disease

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The pivotal role of immune response in the pathogenesis of neurodegenerative diseases, including Parkinson's disease (PD), cannot be overlooked. The aggregation α-synuclein triggers an intricate interplay between innate and adaptive immunity, resulting in persistent inflammation, which is harmful for the dopaminergic neurons. Hence, fine-tuning the immune response holds great promise as a therapeutic intervention for PD, an unsolved pathology with pressing clinical needs. Our ongoing research pursuit to modulate the immune system within the inflamed substantia nigra of a murine model of PD by employing a platform capable of precisely expressing a therapeutic gene. Previous findings from a murine PD model characterized by progressive dopaminergic neuronal loss, α-synuclein aggregate accumulation, and widespread neuroinflammation, underscore the contributions of adaptive and innate immunity to the underlying mechanisms of neurodegeneration. Notably, our studies have revealed that α-synuclein-activated microglia release significant amounts of inflammatory cytokines and oxidative mediators, provoking the invasion of T cells in the substantia nigra. This intricate immunogenic milieu sustains a toxic environment which is detrimental for neurons. With the intention of developing a potentially translatable therapy, we chose to modulate the immune response in an effort to slow down the rate of cell loss. By designing a viral vector that selectively expresses the immune-modulatory cytokine IL-10 in nigral microglia, we have demonstrated the use of IL-10 as a potential curative molecule for PD. The release of IL-10 from microglia contributes to the protection of dopaminergic neurons by exerting a dual effect on both innate and adaptive immunity. In terms of innate immunity, IL-10 promotes the phagocytic activity of microglia, leading to a reduction in aggregate burden, while its impact on adaptive immunity involves an increase in the percentage of regulatory T-cells among the CD4 T-cell population homing the nigra. However, a persistent presence of cytotoxic T-cells and inflammatory markers in

the tissue following IL-10 overexpression suggests that this may be attributed to the constitutive and uncontrolled expression of the transgene. To overcome this significant limitation and further enhance the neuroprotective effect of the treatment, we aimed to incorporate an additional level of control into our therapeutic platform by making the expression of IL-10 dependent on the presence of inflammatory cues released by activated glia or T-cells. Our results demonstrate the reliability of the inducible "on-demand" platform for expressing IL-10 during inflammation, achieving a sufficient concentration to attenuate the inflammation itself, thus providing a valuable anti-inflammatory system. In conclusion, our findings support the potential of modulating the immune response and utilizing IL-10 as a possible curative option for PD, offering valuable insights into the role of inflammation and autoimmunity in the disease progression and onset. The incorporation of an inducible therapeutic platform adds an additional level of control, enhancing the neuroprotective effect and providing a promising avenue for future therapeutic interventions.

P405

Revisiting the outcome of adult wild-type HTT inactivation in the context of HTT lowering strategies for Huntington's disease

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Huntingtin (HTT) lowering strategies are central to the current therapeutic developments for Huntington's disease. If recent studies indicate that age- and cell-type-specific phenotypes are induced by conditional *HTT* knockout, these experiments do not exactly mimic HTT lowering or gene editing conditions, in term of targeted cells and distribution into the brain. Here, we exploited AAV delivery, used in most CNS gene therapy programs and the KamiCas9 gene editing system to gather data on long-term consequences of wild-type *HTT* inactivation in neurons of adult mice and consequently, the feasibility and safety of *HTT* inactivation in these cells. Behavioral analysis as well as neuropathological and single nucleus RNA sequencing (snRNA-Seq) indicated that *HTT* inactivation in 77% striatal neurons and 16% of cortical projecting neurons of adult mice does not induce behavioral deficits and cellular toxicity. Single nuclei RNA-Seq in 11.5-month-old animals show that the profile and proportions of striatal cells are not altered by *HTT* inactivation. A limited number of differentially expressed genes (254 DEG) were identified mainly in SPN (166) and mainly represented by downregulated genes shared between Drd1 and Dr2 neurons. This suggest that inactivation of WT *HTT* in adult striatal and projection neurons has very limited impact and is safe in the long-term.

P406

Dual intravenous and intracerebroventricular administration of AAV-FMR1 vector results in broad brain biodistribution and rescue of Fragile X phenotype in *FMR1* KO mice

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Fragile X Syndrome (FXS) is the most common single-gene form of autism spectrum disorder and intellectual disability affecting 1 in 3,000 males and 1 in 7,000 females. FXS is caused by insufficient levels of FMRP, a protein with many different functions. Due to the range of functions for FMRP, the most promising approach for a comprehensive rescue of the FXS phenotype is to restore FMRP expression. Multiple studies have shown restoration of FMRP in the brain through gene replacement corrects the behavioral phenotype in *FMR1* KO mice. However, efficacy in an animal model of FXS has historically been difficult to translate to clinical benefit. Therefore, we sought to develop an AAV-based gene replacement therapy that would correct the FXS phenotype observed in *FMR1* KO mice in a clinically meaningful way.

Four AAV-FMR1 gene replacement candidates were developed and tested for in vitro expression and in vivo expression and efficacy in *FMR1* KO mice. The optimal AAV-FMR1 candidate showed expression across multiple brain regions when administered by intravenous (IV) or intracerebroventricular (ICV) routes. Highest cortical expression was observed following ICV administration, while IV administration resulted in broad distribution across the brain. Additionally, AAV-FMR1 demonstrated a reduction in audiogenic seizure incidence and score in an audiogenic seizure model with *FMR1* KO mice when delivered by ICV and IV dual route of administration. In a behavioral study to measure anxiety in response to environmental change in mice, we demonstrated that *FMR1* KO mice spend significantly more time digging after nest removal than do wild-type animals. AAV-FMR1 administration was effective in reducing time spent digging when given by ICV or IV injection, suggesting potential for therapeutic efficacy when FMRP is restored to the brain.

In order to better correlate nonclinical efficacy to potential clinical impact, an EEG multi-electrode array (MEA) assessment was adapted for use with *FMR1* KO mice. MEA assessment provides a quantitative preclinical electrophysiology evaluation that may be translated to clinical high density EEG observations in Fragile X patients. A pilot study demonstrated differences in brain activity between WT and *Fmr1* KO mice using MEA assessments. In a subsequent treatment study, we evaluated MEA activity in brain, biodistribution, and protein expression to show that FMRP distribution across the brain resulted in correction of the *FMR1* KO phenotype in brain activity (excessive resting state gamma power) following administration of ICV + IV AAV-FMR1. These findings show gene replacement with AAV-FMR1 is a promising strategy for treatment of Fragile X syndrome.

P407

Restoration of brain cholesterol metabolism as gene therapy approach in Huntington's disease (HD): bilateral striatal administration is needed to elicit therapeutic benefit in HD mice

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In Huntington's disease (HD), unbalanced brain cholesterol homeostasis has a major impact in HD pathophysiology. Cholesterol 24-hydroxylase (CYP46A1), a key neuronal enzyme, is reduced in HD patients' brains. CYP46A1 decreases cholesterol in neurons, regulating cholesterol biosynthesis, and is a key neuronal stress response factor. In HD, reduced CYP46A1 levels are linked with broad cholesterol accumulation in neuronal membranes, impairing vesicular transport, synaptic transmission and clearance of misfolded mutant Huntingtin (mHTT), contributing to neuronal demise (Kacher *et al*. 2022). Restoring CYP46A1 levels in HD mice and re-establishing cholesterol metabolism in striatal neurons using an Adeno-Associated Vector (AAV) , mitigated neuronal dysfunction and mHTT accumulation (Boussicaut 2016; Kacher 2019). Here, we compared motor and neuropathological outcomes of unilateral vs. bilateral striatal administration of AB-1001 (AAVrh10-CYP46A1) to support unilateral or bilateral AB-1001 administration in HD patients, testing two doses. The cylinder and openfield (locomotor activity) tests showed no differences between R6/2 mice administered with AB-1001 unilaterally or bilaterally. Motor balance assessed by rotarod, showed that AB-1001 unilateral administration did not improve motor performance of R6/2 mice. Bilateral AB-1001 administration improved motor performance of R6/2 mice (60%) compared to vehicle-treated R6/2 mice. In mice receiving AB-1001 unilaterally, a substantial aggregate reduction (25-28%) was noticed in injected striatum, with thin non-significant reduction in the contralateral non-injected hemisphere (15-17%). In R6/2 mice administered with AB-1001 bilaterally, a decrease of aggregates (28%) was noticed in both hemispheres. Neurofilament light-chain (NfL) remained unaffected in R6/2 mice receiving AB-1001 unilaterally, though a trend to decline was observed in mice injected bilaterally and in R6/2 mice unilaterally administered with AB-1001 at high titer. These findings support that bilateral AB-1001 administration may be important to obtain the desired clinical therapeutic effect and that unilateral injections are insufficient.

P408

AAV-mediated dual *HEXA-HEXB* gene therapy reverts Sandhoff and Tay-Sachs diseases

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Sandhoff and Tay-Sachs diseases are rare autosomal recessive lysosomal storage diseases resulting from the deficiency of β-hexosaminidase A (HEXA), a crucial enzyme for GM2 ganglioside degradation. HEXA is composed of α and β subunits, encoded by *HEXA* and *HEXB* genes, respectively. Tay–Sachs disease is caused by mutations in *HEXA* gene, while Sandhoff disease results from mutations in *HEXB* gene. Both conditions present severe, progressive neurodegeneration with indistinguishable devastating pathology, and to date, there is no cure. Here, using mouse models of Sandhoff and Tay-Sachs diseases, we demonstrated the therapeutic benefit of a novel gene therapy approach based on a single intra-CSF administration of new AAV9 vectors encoding simultaneously both *HexA* and *HexB* genes (AAV9-*HexA*-*HexB*). AAV-mediated gene transfer resulted in widespread expression of HEXA enzyme in the whole CNS of Tay-Sachs and Sandhoff mice. Additionally, treatment of Sandhoff mice with AAV9-*HexA*-*HexB* vectors resulted in reversion of primary GM2 and secondary cholesterol accumulation, as well as normalization of CNS lysosomal pathology, leading to correction of myelinization, the altered autophagic flux and neuroinflammation. Moreover, intra-CSF delivery of AAV9-*HexA*-*HexB* vectors also efficiently transduced the liver, enabling the secretion of HEXA into circulation, which corrected peripheral GAG and cholesterol storage and lysosomal pathology of Sandhoff disease. These improvements were paralleled to normalization of behavioural deficits, including locomotor alterations, coordination, and mobility. More remarkably, treatment with the dual gene AAV9-*HexA*-*HexB* vector significantly extended lifespan compared to the co-administration of AAV9-*HexA* and AAV9-*HexB* vectors. Taken together, our pre-clinical results demonstrated that a single intra-CSF administration of AAV9-*HexA*-*HexB* vectors led to whole-body correction of both CNS and peripheral pathology, providing solid data for the clinical translation of this approach to treat Tay-Sachs and Sandhoff patients.

P409

Direct Cas9 protein delivery mediated gene editing in the retina

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Genome editing by CRISPR-Cas holds promise for the treatment of retinal dystrophies. For therapeutic gene editing, transient delivery of CRISPR-Cas9 is preferable to viral delivery which leads to long-term expression with potential adverse consequences. Successful delivery of Cas9 protein and its gRNA as ribonucleoprotein (RNP) complex has been reported *in vivo* in the retinal pigment epithelium (RPE) but not in the photoreceptors- the main target of retinal dystrophies. Here, we investigate the feasibility of direct RNP delivery to photoreceptors and RPE cells without adding any carrier compounds. We show that RNPs composed of Cas9/sgRNA or adenine-base editor/sgRNA induce gene editing in retinal cells at variable rates depending on the cell type, genomic location, and the gRNA efficiency. Using a guide RNA targeting *Sag*, a gene highly expressed in photoreceptors, leads to a fifteen-fold improvement of the indels in the neural retina compared to a guide RNA targeting *Vegfa* predominantly expressed in the RPE. Interestingly, cationic lipids which have been shown to facilitate CRISPR/Cas9 RNP delivery into the inner ear cells do not improve CRISPR/Cas9 RNP delivery to retinal cells. RNP delivery at high

concentrations leads to the activation of microglial cells indicating a need to improve delivery efficiency for future therapeutic use.

P410

A New Approach for Treating Epilepsy: CNS-targeted Antioxidant Gene Therapy

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Epilepsy is a common neurological disorder affecting 1% of the global population, significantly burdening patients and society. Although many epilepsies are acquired following brain injury, available treatments only alleviate symptoms, and no effective prophylaxis or cure exists. Accumulating evidence suggests that oxidative stress plays a critical role in the development of seizures and epilepsy and that pharmacological targeting of oxidative stress can prevent spontaneous seizures. However, non-specific antioxidant therapies may disrupt the physiological balance of oxidants/antioxidants, highlighting the need for targeted interventions.

Here, we used AAV vectors to drive the expression of the Nrf2-encoding gene, which promotes the endogenous antioxidant systems, under the control of CaMKIIa, a constitutive, cell-type-specific promoter for targeting excitatory neurons.

We demonstrated that our AAV-CaMKIIa-Nrf2 vectors were selectively expressed in neurons and showed minimal expression in other cell types. Furthermore, our AAV-CaMKIIa-Nrf2 vector significantly decreased neuronal cell death induced by kainic acid-SE in the hippocampus. When injected prior to KA-SE, our AAV-CaMKIIa-Nrf2 vector dramatically reduced seizure frequency over 12 weeks and significantly decreased the total number of seizures compared to control rats. Additionally, 50% of animals remained seizure-free for 12 weeks after SE induction, and only 20% of animals became epileptic after treatment with our AAV-CaMKIIa-Nrf2 vector compared to 100% of animals in the control group.

Our cell type-specific approach for targeted delivery of antioxidant therapies offers a promising strategy for combating oxidative stress following brain injury, preventing or modifying the development of epilepsy, while preserving the critical balance of oxidants/antioxidants in non-affected cells.

P411

Sandhoff Disease is Corrected in a Mouse Model by scAAV9-*HEXM* Gene Transfer

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Sandhoff disease (SD) is an autosomal recessive lysosomal storage disorder characterized by progressive neurodegeneration due to an excessive accumulation of GM2 gangliosides (GM2) in neurons. The resulting widespread neuronal dysfunction eventually leads to death, typically by age 4 in the most common, infantile form. The hydrolysis of GM2 is conducted by a lysosomal enzyme, β-hexosaminidase A (HexA), a heterodimer comprised of an α- and a β-subunit, encoded by genes *HEXA* and *HEXB*, respectively. SD arises from a mutation in the *HEXB* gene leading to the deficiency or absence of β-subunits, and consequently, accumulated GM2. A novel isoenzyme of human HexA has been developed as a hybrid μ-subunit which homodimerizes to form an enzyme named HexM. The effectiveness of HexM *in vivo* has been established in previous studies, which demonstrated improved survival in a *Hexb*$^{(-/-)}$ mouse model of SD following gene transfer of the *HEXM* gene packaged in a self-complementary adeno-associated viral vector, serotype 9 (scAAV9). This study assessed the dose response of a scAAV9-*HEXM* treatment in the *Hexb*$^{(-/-)}$ mouse model with dual delivery via intravenous (IV) and intra-cisterna magna (ICM) routes, utilizing osmotic induced flow, and ancillary administration of immunosuppressants. A total of 10 cohorts (N = 10/cohort), received infusions simultaneously through IV and ICM routes. The IV route had six possible infusates (vehicle, 5 different vector doses) and the ICM route had three (vehicle, 2 vector doses). The study design enables a comparison of treatment efficacy between delivering the total dose through a single route or splitting it between two routes. The researcher who conducted the procedure, behavioural testing, and all analyses was blinded to which infusate each animal received. Behavioural and blood collections were done at specific time points until the mice reached their humane endpoint. Upon termination, tissue and blood samples were collected for analysis of Hex enzyme activity, GM2 accumulation, histology, vector biodistribution, and immune response. Results show up to a >6.5-fold increase in median survival following scAAV9-*HEXM* vector infusion in *Hexb*$^{(-/-)}$ mice in the longest-lived cohort. This cohort survived two years, similar to the heterozygous control group, whereas untreated *Hexb*$^{(-/-)}$ mice only live four months. Additionally, the longest-lived treated cohorts showed consistent behavioural performance over a two-year lifespan with no observable differences to that of the heterozygous control group. Results of the GM2 accumulation assays and the HexM enzyme activity assays echo the survival of the respective cohorts, with significant differences seen in multiple treated cohorts. The greatest reduction in GM2 (>12.9-fold; $p < 0.0001$) and largest increase in Hex enzyme activity (>51-fold; $p < 0.05$) is seen in the longest surviving cohort. Analysis for HexM enzyme activity in tissues, vector biodistribution, and histology will be presented. There is evidence of a strong dose-response and greater than two-year gene expression durability in this novel gene therapy for SD with implications for improved survival and quality of life in a clinical setting.

P412

Regulating transgene expression is essential to avoid toxic effects of supraphysiological GDNF doses in gene therapy for Parkinson's disease

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Glial cell line-derived neurotrophic factor (GDNF) protects nigro-striatal dopaminergic (DA) neurons and reduces motor symptoms when applied in the rodent striatum in toxin-induced Parkinson's disease (PD) models. However, although Phase I uncontrolled clinical trials based on intraputaminal GDNF protein delivery were encouraging, placebo-controlled trials have failed to demonstrate significant clinical benefits.

Our hypothesis is that GDNF beneficial effects are dose dependent.

We have previously described a doxycycline (Dox)-regulated AAV vector allowing to adjust GDNF dose at clinically acceptable Dox doses. Using different Dox and vector doses, we have administered GDNF at concentrations ranging from 3X to 20X the endogenous level in the striatum of unilaterally 6-hydroxydopamine-lesioned female Wistar rats. The rats were Dox-treated for 17 weeks either continuously or intermittently for 2 weeks with 2 weeks interruptions. Behavioral tests revealing motor impairments were amphetamine-induced rotations and drug-free distance run and rotations in an Open field. Output measures were: DA neurons survival and cell size, DA fibers quantification, 8-oxo-2'-deoxyguanosine immunostaining (oxidative stress).

Significant reduction of the motor impairments and restoration of striatal DA neurons innervation were observed at GDNF tissue concentrations of 3X and 10X. In contrast, at the highest GDNF dose (20X), motor deficits were not reversed, and the number of DA neurons and their striatal DA innervation were not increased relative to untreated rats. Strikingly, DA neurons harbored a higher level of DNA oxidation as compared to the lowest GDNF dose. When the treatment was applied intermittently, the highest GDNF dose also induced significant improvements, increased striatal re-innervation, and did not induce increased levels of DNA oxidation.

It is known that DA neurons with larger soma and wider terminal arborization are more susceptible to degeneration in PD patients and in animal models. This preferential degeneration of the larger neurons results in a decreased mean cell size. We compared the mean cell area in the SNpc in the control group versus the GDNF-treated groups. We found that only the GDNF 3X treatment increased the mean cell size. In contrast, in the GDNF 20X and GDNF 20X Int groups, the mean cell size was similar to that of the untreated group.

Finally, we evaluated aberrant DA sprouting in the substantia nigra pars reticulata, in which transduced striato-nigral neurons project and deliver GDNF ectopically. In the GDNF 20X group and to a lesser extent in the GDNF 20X Int group, ectopic dopaminergic fibers were observed. In contrast the GDNF 3X treatment did not result in aberrant sprouting.

In conclusion the GDNF-3X treatment was therapeutic according to all parameters by restoring DA neurons number and size as well as striatal innervation whereas, the 20X treatment restored none of these parameters. The GDNF 20X Int group restored DA neurons numbers and striatal innervation but not mean cell size.

Only the GDNF 3X group showed no undesirable effects (oxidative stress or aberrant sprouting).

In future clinical trials, it will be important to control GDNF administration to avoid overdosage potentially reducing the clinical benefits due to toxicity.

P413

Changes of the cortical and subcortical networks following the gene therapy for aromatic l-amino acid decarboxylase deficiency

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Aromatic l-amino acid decarboxylase (AADC) deficiency, a rare autosomal recessive disorder caused by mutations in the *dopa decarboxylase* (*DDC*) gene, presents with cognitive and motor impairment, including dystonia and oculogyric crisis (OGC) due to impaired synthesis of dopamine and serotonin. Adeno-associated virus (AAV) vector-mediated delivery of *DDC* into the putamen has shown the long-term synthesis of AADC enzyme and improved cognitive and motor functions, the disappearance of dystonia, and decreased OGC. Our previous studies revealed that motor recovery after gene therapy correlated with the volume of the highly transduced putaminal area that was dominantly connected to the prefrontal area of the fronto-parietal control network (Onuki et al., 2021). However, it remained to be elucidated how gene therapy changed the connectivity of the cortical and subcortical networks. We examined eight patients with AADC deficiency who received putaminal AADC gene therapy. The transduction was assessed by the high-resolution positron emission tomography with a specific AADC tracer, 6-[18F] fluoro-l-*m*-tyrosine (FMT-PET). The functional connectivity across cortical and subcortical areas was calculated from the resting-state functional magnetic resonance imaging (fMRI). The FMT-PET data were acquired from baseline to five years. For resting-state brain activity, fMRI data were obtained from two patients from baseline to one year post-treatment, and from one patient from baseline to two years post-treatment. In addition, resting-state brain activity data were acquired from one patient at two years post-treatment and from four patients at five years post-treatment. FMT-PET results confirmed the robustly increased FMT uptake from baseline to five years post-treatment in the putamen and the substantia nigra. The functional connectivity analysis of the resting-state fMRI from the baseline to six months post-treatment in two patients revealed that the organized patterns within the fronto-parietal control network were expressed after treatment. Additionally, the functional connectivity analysis across subcortical regions showed increased connectivity between pars compacta (SNc) and pars reticulata (SNr) of the substantia nigra after the treatment. This organized pattern of the prefrontal areas and the upward trend in interregional connectivity of SNr and SNc were also observed in other patients after the gene therapy. Our results suggest that the AADC gene therapy to the putamen may have affected the functional connectivity in the cortical and subcortical network. The expressed organized network pattern of the fronto-parietal control network may reflect the cortical development driven by the dopaminergic facilitation by gene therapy. Since SNr neurons display local axon collateral networks that connect to SNc neurons, the increased interregional connectivity may be derived from the dopaminergic recovery in the basal ganglia network. These changes in the cortical and subcortical networks are probably the basis for therapeutic effects, including the improvement of both cognitive and motor function.

P414

Gene therapy against amyotrophic lateral sclerosis: a bicistronic AAV vector for RNAi against mutated SOD1

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Amyotrophic Lateral Sclerosis (ALS) is a fatal motor neuron disease that results in the progressive loss of motor neurons controlling voluntary movements. Although the mechanisms of motor neurons degeneration remain elusive, in some inherited and sporadic forms of ALS the misfolding and aggregation of Superoxide Dismutase 1 (SOD1) lead to the gain of toxic function. Our therapeutic approach is based on AAV gene therapy to reduce the level of SOD1 expression by RNA interference (RNAi), and thereby limit the toxic effects of the misfolded protein. To maximize treatment efficacy, we designed a novel gene therapy platform for co-targeting neurons and astrocytes in the spinal cord via the injection of a single AAV9 vector expressing a miRNA against the human SOD1 mRNA (miR SOD1). This bicistronic vector contains two cassettes controlled by CNS-specific promoters driving expression in astrocytes (gfaABC$_1$D promoter) and neurons (hSynapsin1 promoter). Intrathecal injection of AAV9-bicistronic-miR SOD1 in 5 months-old high-copy SOD1 G93A ALS mice (a well characterized mouse model of ALS) decreased SOD1 in spinal cord tissue at both mRNA and protein levels, increased neuromuscular occupancy and improved muscle function and motor performance. In addition, intracerebroventricular (ICV) injection of this vector in early post-natal mice prolonged the median survival from 156 days to more than 300 days, depending on the vector dose administered.

In non-human primates (NHP), the intrathecal injection of the AAV9-bicistronic-miR SOD1 vector led to a broad distribution of the vector DNA and miR SOD1, along the entire spinal cord. In situ hybridization showed a detectable presence of vector DNA and miR SOD1 in more than 70% of the neurons in the lumbar spinal cord. Depending on the number of vector copies present in motoneuron nuclei, the level of endogenous SOD1 mRNA silencing in neuronal cells reached 80%. The expression of miR SOD1 was also detected in cells with astrocytic morphology. Importantly, although a high number of vector copies was found in liver tissue, the expression of miR SOD1 remained at low level in this tissue and SOD1 mRNA expression was not decreased.

Overall, these results demonstrate the advantage of using a bicistronic vector combining neuron- and astrocyte-specific promoters to deliver a microRNA into key CNS cell types. This vector system is an effective platform to deliver RNAi against SOD1 and other CNS targets, as a disease-modifying therapy against ALS.

P415

Shank3 gene activation mediated by zinc finger transcriptional activators (ZF-A) as a therapeutic approach for Phelan-McDermid Syndrome

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Phelan-McDermid syndrome (PMS) is a rare genetic condition characterized by clinical features with varying severity, including intellectual disability, absent or delayed speech, and autism spectrum disorders (ASD). PMS is caused by a deletion or structural change in chromosome 22 or a pathogenic variant of the *SHANK3* gene. *SHANK3* encodes a synaptic protein localized at excitatory synapses to organize scaffolding proteins crucial for proper synapse formation or dendritic spine maturation. Mutations or loss of a *SHANK3* allele due to copy number variations can lead to SHANK3 haploinsufficiency causing synaptic and circuitry deficits associated with PMS. Thus, we hypothesized that selectively upregulating *SHANK3* gene expression, using our zinc finger transcriptional activator (ZF-A) platform, will restore physiological levels of SHANK3 protein critical for normal neural circuit function. To test this hypothesis, we designed ZF-As targeting the mouse *Shank3* gene and assessed *Shank3* mRNA and SHANK3 protein levels in cultured mouse cortical neurons. We identified several potent and highly specific ZF-As, producing zero to minimal off-targets, able to upregulate *Shank3* mRNA, and restore SHANK3 protein to normal levels *in vitro* in primary neurons derived from both wildtype (WT) mouse and a mouse model of PMS with heterozygous *Shank3* mutations (*Shank3*$^{delta4\text{-}22}$). Further, we performed *in vivo* studies using intravenous, AAV-mediated ZF-A delivery in WT mice. We confirmed AAV-ZF-A dose-dependent upregulation of *Shank3 in vivo* in relevant brain regions and cell subtypes by bulk tissue and single-cell mRNA and protein expression analyses. Thus, our results support the development of ZF-As to specifically restore SHANK3 to physiological levels, providing a potential precision therapy for PMS. In addition, these data highlight the utility of ZF-As in potentially treating haploinsufficiency disorders.

P416

Innovative single gRNA enhanced-deletion genome editing-based correction of aberrant splicing due to pathogenic deep-intronic variants

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Although CRISPR/Cas9 editing has been successful in correcting aberrant splicing by targeting sequences involved in the faulty process, the standard approach of generating a deletion that contains the relevant pathogenic deep intronic variant and intronic sequences retained upon

mis-splicing has several drawbacks. These include the induction of higher activation of P53-related pathways when using multiple gRNAs, the increased risk of off-target effects and chromosomal rearrangements, and the requirement of additional components for delivery. To overcome these issues with standard CRISPR/Cas9 approaches, we engineered Enhanced-Deletion Cas (EDCas) molecules: EDSpCas9 and an AAV-packable ED-synthetic RNA-guided endonuclease (EDsRGN). By using only one gRNA, EDCas molecules generate larger deletions at the targeted sites, inducing significant perturbation of sequences involved in the faulty splicing process. This in turn prevents their recognition by the splicing machinery, resulting in consistent splicing correction.

In one of our current projects, we demonstrate that EDCas molecules are effective in correcting aberrant splicing caused by the *USH2A*:c.7595-2144A>G deep-intronic variant in both minigene assays and patient-derived fibroblasts. This pathogenic variant activates a cryptic splice site, resulting in intron (pseudoexon) retention in the mature mRNA transcript, which causes frame-shift and pre-termination codon formation. Using six single different gRNAs coupled to EDSpCas9, we achieved higher splicing rescue rates (53.5±11.8% - 88.0±1.9%) compared to SpCas9 (34.0±25.3% - 71.8±30.0%) in HEK293T cell minigene assays. Four lead gRNAs were further validated in patient-derived *USH2A*:c.7595-2144A>G homozygous fibroblasts, showing high splicing rescue rates (85.7±3.7% - 92.4±4.8%), consistent with the minigene assay results. The genomic deletion profiles obtained clearly demonstrate that EDSpCas9 is capable of inducing enhanced and directional deletions compared to SpCas9, as evidenced by the deletion frequency across the four leading gRNAs (with a size of ≥15 bp: 61±16% for EDSpCas9 versus 8±5% for SpCas9). EDsRGN was then used to preliminary assess the splicing rescue potential of 3 selected gRNAs in two different cell lines: HEK293T and ARPE19. Also, in this case, the results consistently demonstrated higher splicing rescue efficacy for EDsRGN across both cell lines. To optimize the design of AAV-EDsRGN particles, small promoters were tested in HEK293T and RP1 cells by lentivirus transduction, identifying EF-1α and CMV minimal promoters as best candidates for subsequent AAV production.

The use of engineered EDCas molecules to address splicing defects is showing promising preclinical results transferable to advanced disease models. Ongoing experiments include assessing potential off-target effects (including chromosomal translocations, which have been shown by others to be drastically reduced when implementing a comparable gene editing approach), the effect on the activation of P53-related pathways, and the implementation of EDsRGN to allow AAV-mediated delivery of the editing system. In addition, the correction of aberrant splicing caused by exonic and intronic pathogenic variants is being explored. Ultimately, by utilizing similar EDCas molecules coupled to only a single gRNA, we are developing mutation-independent genome editing strategies able to address up to 40% of all the genotypes of the highly heterogeneous mutational spectrum of the *RHO* gene (the most commonly mutated gene in autosomal dominant Retinitis Pigmentosa).

P417

Functional and molecular evaluation of intravitreal (IVT) AAV gene therapy vectors for the treatment of Geographic Atrophy (GA)

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Dry age-related macular degeneration (Dry AMD) with geographic atrophy (GA) is a highly prevalent disease, characterized by retinal pigment epithelium (RPE) and photoreceptor death leading to vision loss, which affects the quality of life in the aging population worldwide. Although the cause of Dry AMD is unknown, activation of components of the complement cascade have been associated with GA. Complement Factor I (CFI) is a rate-limiting enzyme in the complement cascade, naturally inhibiting the activity of proteins involved in complement overactivation. Therefore, continuous overexpression of CFI in the ocular tissue has the potential to inhibit the complement cascade in the eye, halt GA lesion growth and preserve vision in patients with Dry AMD. This can potentially be achieved by intravitreal (IVT)-delivery of adeno-associated viral (AAV) vectors engineered for broad retinal transduction and CFI expression. The ability to administer AAV vectors to patients via IVT delivery, a routine in-office procedure, is ideal for highly prevalent ocular disorders such as Dry AMD. In order to generate an AAV vector to treat patients with GA, we developed an improved codon-optimized CFI human cDNA (CFIco) sequence, which improved CFI expression over the wild-type sequence. AAV-CFIco was packaged into two of Adverum's proprietary highly retinotropic serotypes - AAV2.7m8 and AAV2.5T-LSV1 - which have shown to successfully transduce retinal cells following IVT delivery. Next, we performed a study using non-human primates (NHP). In this study, NHP subjects were administered bilateral IVT injections of AAV2.7m8-CFIco or AAV2.5T-LSV1-CFIco at either 3E10 vector genomes/eye (vg/eye) or 1E11 vg/eye (n=3/group), or vehicle (n=3/group). Vitreous humor (VH) was collected for CFI quantification on days 28, 62 and 88 post dose. Ocular tissues were analyzed for vg and mRNA biodistribution. Administration of AAV2.7m8-CFIco and AAV2.5T-LSV1-CFIco at both doses resulted in production of CFI. Mean peak CFI levels at the 3E10 vg/eye dose were 2464 ng/mL for AAV2.7m8-CFIco and 575 ng/mL for AAV2.5T-LSV1-CFIco. At the 1E11 vg/eye dose, mean peak CFI levels were 2313 ng/mL for AAV2.7m8-CFIco and 557 ng/mL for AAV2.5T-LSV1-CFIco. Overall, IVT administration of AAV2.7m8-CFIco and AAV2.5T-LSV1-CFIco in NHP subjects was well tolerated. No adverse systemic clinical signs were observed during the 3-month study. Animals had dose-dependent and self-resolving slight to mild ocular inflammation characterized by pigment and cells in the vitreous, and no abnormalities of the anterior segment or lens were found. In summary, the findings from the NHP study suggest IVT administration of either AAV2.7m8-CFIco or AAV2.5T-LSV1-CFIco at doses as low as 3E10 vg/eye (human equivalent dose of 6E10 vg/eye) may be capable of providing sustained therapeutic levels of CFI for the treatment of Dry AMD patients with GA. Importantly, the IVT route of administration allows for the routine in-office administration, something critical for this highly prevalent disease.

P418

Empowering a novel regulatory code for retinal gene therapy

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Rhodopsin-Autosomal dominant Retinitis Pigmentosa (RHO-ADRP) is a progressive photoreceptor degeneration leading to an incurable loss of vision. To treat this blinding inherited disease, we established a solid gene therapy proof of principle with Adeno-associated virus (AAV) vectors, which couples two approaches, gene silencing with gene replacement (S&R) in a single AAV vector. For the silencing step we used an unconventional transcriptional repression system, in which the exclusive use of a DNA-binding protein (ZF-RHO-DNA-BP) blocks Rhodopsin (RHO) expression without the aid of repressor domains binding to a short and accessible DNA Cis

Regulatory Element (CRE) of the RHO-proximal promoter region. We exploited RHO own regulatory grammar to fully regulate both the gene therapy modulator, ZF-RHO-DNA-BP and the RHO replacement cassettes. Thus, we studied this CRE with higher resolution by changing the number, orientation, order, spacing and their sequence composition demonstrating a wide range of modulation of the transcriptional outputs. In this way, we obtained specific promoter elements with different strengths that have enabled to empower the single AAV vector construct with balanced transcriptional silencing and replacement of RHO using lower doses of AAV vectors than previously shown.

P419

Recombinant adeno-associated virus (rAAV)-based gene therapy for Lafora disease

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Lafora disease is a rare and fatal form of progressive myoclonic epilepsy that usually appears early in adolescence. Patients present with myoclonus, neurological deterioration, and generalized tonic-clonic, myoclonic, or absence seizures. Symptoms worsen until death, usually within the first ten years of clinical onset. The main neuropathological feature is the presence of abnormal polyglucosan inclusions positive to periodic acid-Schiff (PAS), known as Lafora bodies (LBs), in the central nervous system and other tissues. Mutations in two genes, *EPM2A*, which encodes laforin, a dual-specificity phosphatase, or *EPM2B*, coding for malin, an E3 ubiquitin ligase, are responsible for the disease. Currently, there is no effective treatment available. In this study, we evaluated a rAAV-based gene therapy in the *Epm2a*$^{-/-}$ mouse model of Lafora disease, which mimics some of the main symptoms present in patients, including the presence of LBs, neurodegeneration, neuroinflammation, epileptic activity, and cognitive and motor impairments. We used a novel rAAV vector containing the human *EPM2A* gene (rAAV2/9-CAG-h*EPM2A*). To assess the effectiveness of this therapy, we conducted the object recognition task (ORT) to evaluate episodic memory, the rotarod to assess motor coordination, the actimeter to measure spontaneous locomotor activity, and video-EEG recordings to analyze epileptic activity. We administered the epileptogenic agent pentylenetetrazol (PTZ) to analyze neuronal hyperexcitability. Neuropathological features were evaluated by immunohistochemistry with the NeuN antibody to assess neurodegeneration and the GFAP antibody to analyze neuroinflammation, and by PAS-diastase staining to evaluate LB formation. Proteomic and phosphoproteomic analysis were performed to investigate potential changes in molecular pathways in response to the human laforin. Three and nine months after a single intracerebroventricular injection in 3-month-old *Epm2a*$^{-/-}$ mice, we observed that rAAV therapy ameliorated most of the neurological alterations. Specifically, rAAV2/9-CAG-h*EPM2A* delayed the onset of cognitive and motor impairments, enhanced motor coordination, and reduced epileptic activity. Neuroinflammation and LB formation were also decreased. Proteomic and phosphoproteomic analysis indicated beneficial changes in certain molecular pathways altered in Lafora disease, such as downregulation of glycogen metabolism and proteins involved in neuronal excitability or upregulation of proteins involved in protein folding. Moreover, rAAV-based laforin replacement produced relevant changes in the phosphorylation pattern of several proteins implicated in

different molecular pathways such as mTOR or RAS signaling pathway which are related with many cellular events including neuronal excitability. We conclude that gene replacement therapy using ICV injections of rAAV2/9-CAG-h*EPM2A* is a promising treatment for Lafora disease.

P420

Alpha-synuclein lowering and rescue of motor phenotype by miRNA-based AAV gene therapy in an *in vivo* Parkinson's disease rat model

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Parkinson's disease (PD) is a progressively debilitating neurodegenerative disease with an increasing prevalence with age. Only symptomatic therapies are available which do not tackle the underlying disease mechanism. One of the underlying causes of PD is aggregation of alpha-synuclein protein (α-syn), encoded by the SNCA (Synuclein Alpha) gene. Aggregated α-syn is one of the main components of Lewy Bodies (LBs), a key neuropathological hallmark in PD. Typically, LB pathology originates in brainstem and extends to midbrain and cortical regions with disease progression, in parallel with neurodegeneration of nigro-striatal dopaminergic circuits and other neurotransmitter systems. Our hypothesis for a disease modifying therapy is that lowering α-syn protein levels in relevant brain regions may reduce aggregation and degeneration, ultimately halting disease progression.

We are developing an adeno-associated virus (AAV) gene therapy to deliver SNCA-targeting microRNAs (miSNCAs) engineered to decrease α-syn mRNA and protein levels. We have previously presented data on design and in vitro selection of miSNCA candidates targeting all human SNCA splicing variants. Here, we evaluate the potential phenotypic improvement of AAV5-miSNCA in an α-syn rat PD model. Adult rats received AAV1/2 overexpressing human A53T-α-synuclein ipsilaterally in the substantia nigra, at a dose known to cause neurodegeneration and PD-like motor phenotypes. Two weeks after AAV1/2-hA53T-α-synuclein administration, three AAV5-miSNCA candidates were injected in the substantia nigra. All tested AAV5-miSNCA candidates showed correct microRNA processing and target engagement at both mRNA and protein levels. Moreover, dopamine metabolite deficiencies and motor deficits in hA53T-α-syn rats were corrected by AAV5-miSNCAs. Additionally, dopaminergic cell loss was rescued in the miSNCA-treated groups as compared to the controls. These results support the potential therapeutic value of α-syn RNAi-based gene therapy for disease modification in PD.

P421

An investigational gene therapy for the treatment of patients with GA secondary to AMD and variants in *CFH*

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Age-related macular degeneration (AMD) is the leading cause of blindness amongst the elderly in the industrialised world and affects millions of individuals globally. Geographic atrophy (GA) is an advanced form of AMD that leads to irreversible loss of vision through development of atrophic lesions of the outer retina, including loss of photoreceptors (PR), retinal pigment epithelium (RPE) and the underlying choriocapillaris. Histological and genetic evidence have emerged implicating chronic local inflammation and activation of the complement system in the pathogenesis of AMD, with genome-wide association studies (GWAS) confirming an association with the complement alternative pathway (AP). The AP regulatory proteins, factor H (FH; *CFH*) and factor I (FI; *CFI*), work synergistically to control amplification of the AP, and rare genetic variants in *CFI* and *CFH* and common SNPs in *CFH* are amongst the highest risk factors for AMD. Supplementation of FH at the site of disease, therefore, holds therapeutic potential to slow the progression of AMD and onset of GA by dampening overactive complement, with potential for highest efficacy in populations with rare and common genetic variation in *CFH*. Here, we explore supplementation of human Factor H-like protein 1 (hFHL-1), an alternative splice variant of *CFH*, from a recombinant adeno-associated viral vector (rAAV) as a potential gene therapy approach for GA secondary to AMD. First, wild type (WT) and codon-optimised (co) hFHL-1 cDNA sequences were vectorised, and *in vitro* transduction confirmed expression from both vectors, with higher levels of expression observed from co hFHL-1. Furthermore, cofactor assays of C3b cleavage and real-time analysis of AP C3 convertase control by surface plasmon resonance revealed expression of fully functional hFHL-1. The co hFHL-1 sequence was then selected for progression *in vivo*. Subretinal injection of a rAAV vector encoding co hFHL-1 in rodents led to detectable levels of secreted hFHL-1 in ocular fluids, as well as a significant reduction of CNV lesion size in the mouse laser-induced choroidal neovascularisation (CNV) model of AMD. Collectively, the pre-clinical data provided here demonstrate the therapeutic potential of FHL-1 in slowing disease progression of AMD by limiting complement-mediated inflammation in the posterior segment of the eye, supporting its development as a potential therapy for the treatment of GA secondary to AMD.

P422

Vertebrate cone opsins as a highly sensitive optogenetic tool for second-generation vision restoration

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Microbial opsins (light-sensitive ion channels and pumps) 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 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 signaling in mouse RGCs, we expressed short-wavelength sensitive mouse cone opsin (Opn1sw) in the same cells using recombinant AAV vectors. 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 signaling pathway. Single cell RNA sequencing in RGCs of both degenerated (rd1) and healthy (C57BL/6) mice showed no significant differences in G-protein-expression landscape. This consolidates our hypothesis that different cone opsins might activate different G protein pathways in the same cell type, triggering different types of responses through different effectors. Overall, our results indicate a need to better understand the intricate relationship between cone opsins and their signaling cascades within different cells. Such understanding will lead to the second-generation therapies using such opsins.

P423

Generation of lentiviral-based mouse models of spinocerebellar ataxias: where to target?

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Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), with a prevalence of 1 to 5 in 100 000 individuals, is the most common autosomal dominantly-inherited ataxia worldwide. It is caused by an unstable abnormal CAG expansion in exon 10 of the *ATXN3* gene. The resulting non-functional ataxin-3 protein product carrying a polyglutamine stretch, is the main responsible for the neuropathology of the disease. With ataxia being the most prominent clinical feature, over the course of the disease different brain regions are affected, with the hindbrain structures, such as cerebellum and pons, being the most affected in SCA3 patients.

Current animal models fail to recapitulate the pattern of degeneration observed in humans, either due to restricted expression of mutant ataxin-3 to some neuronal cells, or specific brain regions, or due to its overexpression in non-detrimental regions for SCA3 progression. Consequently, when developing advanced therapies, it becomes extremely hard to translate therapeutic outcomes.

To overcome that, in this work we aimed at understanding the contribution of different hindbrain regions in the progression of SCA3 to aid the development of more robust mouse models.

In the present work, lentiviral vectors were used to individually deliver mutant ataxin-3 with 72 glutamines into five distinct hindbrain regions of four-week-old wild-type mice. These regions included cerebellar lobule IV/V, VIII and IX, deep cerebellar nuclei and pons. For each injection site, a control group with lentiviral vector buffer was used to discard injection bias. The motor performance was evaluated using a battery of behavioral tests, including the rotarod and beam walking tests, over a three-month period following disease induction. Neuropathology was assessed at the end of this period.

Depending on the injection site different neuropathological and motor abnormalities were observed. Notwithstanding, lentiviral mediated expression of mutated ataxin-3 led to the formation of ataxin-3 aggregates in all the different targeted regions, as well as astrogliosis, gliosis, and loss of white matter in mouse cerebella.

Overall, our results reinforce the importance of understanding which brain structures need to be targeted to induce disease progression and phenotype. This understanding is crucial to assess the effectiveness of novel therapies.

P424

Regulatory T cells to the rescue in multiple sclerosis: a toxic relationship with brain barriers

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In multiple sclerosis (MS), FOXP3$^+$ regulatory T cells (Tregs) are intrinsically unable to control the autoimmune response directed at the central nervous system. This may impact the success of autologous Treg therapy, which is currently under investigation for treatment of many autoimmune diseases, including MS. In light of this, our recent findings indicate that the inflamed blood-brain barrier (BBB) affects human Treg stability. Using functional *in vitro* assays, we found that the suppressive capacity of migrated Tregs was affected. Transcriptome analysis identified that migrated human Tregs of healthy donors and MS patients have a pro-inflammatory Th1/17 signature and upregulate the mTOR signalling pathway. *In vitro* treatment of migrated Tregs with the clinically-approved mTOR inhibitor rapamycin restored the loss of suppressive function. Next to their textbook immunosuppressive function, Tregs were recently described to have non-canonical regenerative functions as well. Indeed, migrated Tregs express less of the regenerative protein amphiregulin (AREG). In addition, our most recent results now show that Tregs are crucial for brain barrier function, since Treg depletion in wildtype mice causes leakage of both the BBB and the blood-cerebrospinal fluid barrier (BCSFB) at the choroid plexus (CP). IL-34 is produced by Tregs and its receptor, CSF-1R, is present on CP epithelial and BBB endothelial cells. Our *in vitro* data indicates that IL-34 treatment maintains barrier integrity of both CP epithelial and BBB endothelial cells, even in the inflammatory conditions. In line with a defective suppressive capacity in MS, Tregs of MS patients significantly produce less IL-34 compared to healthy donors. Treatment of experimental autoimmune encephalomyelitis (EAE) mice with IL-34 decrease barrier leakage both at the BBB and the BCSFB. These insights and specific target identification can help in significantly improving the efficacy of autologous Treg therapy of MS.

P425

Investigating routes of administration and AAV serotypes for development of a gene-based therapy for spinocerebellar ataxia type 3 (SCA3)

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Spinocerebellar ataxia type 3 (SCA3), also known as Machado-Joseph disease (MJD), is an autosomal dominant neurodegenerative disease caused by an abnormally long CAG tract in the coding region of the *ATXN3* gene. This results in translation of an aberrant polyglutamine chain, which confers toxic properties to the ATXN3 protein leading to neuropathology. Given that different brain regions are affected in SCA3, broad and efficient distribution of a gene therapy to the main disease-affected areas is desirable. In the current study we aimed at determining the best route of administration, i.e., localized intra-parenchymal delivery into the deep cerebellar nuclei (intra-DCN) or broad intra-CSF delivery. Moreover, we explored the transduction potential of two different AAV serotypes (AAV9 and AAV5), encoding an artificial microRNA against mutant *ATXN3* and the reporter gene green fluorescent protein (GFP), in a transgenic mouse model of SCA3 that expresses the full human mutant *ATXN3* gene and recapitulates important disease features. Our results revealed that, independently from AAV serotype, intra-DCN administration resulted in higher number of viral genome copies and greater expression levels of the reporter gene GFP in the target brain regions, mainly in the cerebellum and brainstem. In particular, intra-DCN AAV9 administration led to significantly higher levels of GFP, revealed through qPCR and immunofluorescence data in the previously mentioned target regions. Importantly, our data also demonstrated target engagement in relevant disease-affected regions, illustrated by a trend for reduction of ATXN3 mRNA and protein levels, mainly in animals injected with AAV9 via the intra-DCN route. Altogether, these results provide evidence of the potential of AAV9 transduction and efficient gene silencing in disease-affected areas and constitute a strong basis for the development of AAV9-based therapy for SCA3.

P426

Gene transfer of *WDR45* restored ferritinophagy in SENDA/BPAN patient-derived cells

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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*, a core autophagy-related gene that encodes WIPI4. Since no other variants of core autophagy-related genes show abnormalities in iron metabolism, the pathophysiology of the disease, particularly the function of *WDR45*/WIPI4 in iron

metabolism, remains to be elucidated. Ferrous iron is highly reactive, so the iron homeostasis is tightly regulated. Transferrin receptor and divalent metal transporter 1 (DMT1) are responsible for intracellular iron uptake, and ferroportin (FPN) is responsible for iron export. Excess iron is stored in ferritin as ferric iron in a non-redox and stable state. When the demand for ferrous iron increases in the cell, ferritin is degraded by autophagy, and the ferrous iron is released into the cytoplasm. The ferritin-specific autophagy is called ferritinophagy, wherein a cargo receptor called nuclear receptor coactivator 4 (NCOA4) binds ferritin and directs ferritin to degradation. We evaluated the autophagy and iron metabolism in patient-derived fibroblasts to elucidate the pathophysiology and develop therapeutic options for SENDA/BPAN. The fibroblasts were infected with 5×10^5 vg/cell of adeno-associated virus vectors expressing *WDR45* (AAV-CMV-WDR45). In the patient-derived cells, the expression of WIPI4 and NCOA4 was markedly reduced, and ferritin and ferric iron accumulated. In addition, DMT1 was upregulated, while FPN was downregulated. The oxygen consumption rate was decreased because of mitochondria dysfunction based on ferrous iron loss. Three days after the infection of AAV-CMV-WDR45, the expression of WIPI4 and NCOA4 was restored, and the ferritin levels were reduced in the patient-derived cells. In addition, the expression level of DMT1 and FPN was improved, suggesting that *WDR45* replacement normalised the iron metabolism. Our study reveals that the pathophysiology of SENDA/BPAN involves ferrous iron insufficiency via impaired ferritinophagy through the reduction of NCOA4 expression. Gene transfer of *WDR45* recovered the ferrtinophagy and ameliorated the dysfunction of iron transport. An *in vivo* gene delivery study on *WDR45* deficient mice is underway using AAV9 variant vectors.

P427

AAV-based gene therapy for hereditary spastic paraplegia type 5

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Hereditary spastic paraplegia type 5 (SPG5) is an ultra-rare, recessively inherited neurodegenerative disease caused by bi-allelic loss-of-function mutations in CYP7B1. CYP7B1 encodes the oxysterol 7-α-hydroxylase, which is responsible for metabolising 25- and 27-hydroxycholesterol (HC) in the alternative bile acid pathway. The accumulation of hydroxycholesterols in the liver and serum of SPG5 patients occurs while cholesterol degradation and bile acid synthesis remain largely unaffected due to the intact primary pathway. Notably, hydroxycholesterols can readily cross the blood-brain barrier, leading to elevated levels of HC in the cerebrospinal fluid (CSF) of patients and brain tissue of CYP7B1 knockout (KO) animals. The neurotoxic properties of HC contribute to the characteristic neurological symptoms of SPG5, including progressive spasticity, lower limb weakness and peripheral neuropathy.

Initial attempts to restore HC metabolic activity in the liver by an AAV-based gene replacement therapy using a liver-specific AAV8 construct resulted in highly efficient transduction of liver tissue without detectable hepatotoxicity. While the HC levels in serum were readily normalized already several days after AAV injection and stayed on wildtype level until the endpoint, there was no relevant reduction in brain tissue. Our study showed that CYP7B1 expression in the liver alone is not sufficient to restore wild-type HC levels in the CNS of our mouse model. To overcome this limitation, our follow-up study aims to target both the liver, the primary source of HC, and the

brain, the affected organ in SPG5. By adopting a dual targeting approach, we aim to comprehensively restore HC homeostasis.

In addition to the *in vivo* proof-of-concept study, we will employ induced pluripotent stem cell (iPSC)-based models to investigate potential toxic effects of CYP7B1 overexpression in various neural cell types. This analysis will provide valuable insights into the safety and efficacy of our proposed therapeutic strategy, as well as a better understanding of the disease pathology of SPG5.

By combining targeted intervention in both the liver and the brain, our study aims to address the challenges associated with restoring HC metabolism in SPG5. The results of this study may pave the way for novel therapeutic approaches to the treatment of this neurodegenerative disease.

P428

ICG-mediated photoporation of the ILM to enhance retinal ganglion cell delivery

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Glaucoma is a leading cause of irreversible blindness worldwide. Its primary hallmark is the progressive loss of retinal ganglion cells (RGCs) which inevitably leads to vision loss. A revolutionary cure for glaucoma could be the replacement of these lost neurons with stem-cell derived RGCs.

A mayor challenge to RGC transplantation is the limited migration of the transplanted cells into the retina due to the presence of the inner limiting membrane (ILM). This ILM represents a double-edged sword for RGC engraftment: on the one hand it greatly hinders cell migration into the retina, whereas on the other hand its presence is necessary for correct development of the transplanted RGCs. Photoporation of the ILM offers a controllable method which could allow us to tune the integrity of the ILM to the needs of the donor RGCs.

To photoporate the ILM, we applied the photosensitizer indocyanine green (ICG) (0,1 mg/ml to 1 mg/ml) at the ILM side of bovine retinal explants followed by laser treatment (800 nm, 7 nanosecond pulses) with varying laser fluences and scanning patterns. Next, retinal explants were stained for laminin, enabling examination of ILM integrity using microscopy. IMARIS software was applied to quantify the area per ILM fragment for the various conditions. To explore the impact of ILM photodisruption on RGC transplantation, hESC-derived RGCs expressing TdTomato (20.000 per 5 mm explant) were co-cultured for 7 days on untreated and photoporated bovine explants. On day 7, samples were fixed and stained for Human Nuclei to identify the RGCs, allowing us to quantify the number of RGCs per explant using confocal microscopy.

IMARIS analysis revealed that the average area per ILM fragment of the untreated explants (1163 ± 739 μm^2) was significantly higher compared to photoporated explants (313 ± 560 μm^2), indicating fragmentation of the ILM with 0.5 mg/ml ICG and 1.6 J/cm^2.

Remarkably, RGC transplantation in retinal explants undergoing full ILM ablation was found to be enhanced, seeing that the mean survival of the photoporated samples (0.1 mg/ml ICG; 0.43 J/cm^2) was 2.9 ± 1.8 % compared to 1.5 ± 0.9 % for the untreated samples.

Interestingly, microscopy on flatmounts revealed we were also able of creating a distinct perforated pattern when combining our laser pattern design with 1 mg/ml ICG and 1,6 J/cm^2 fluence, demonstrating the high spatial control of ILM photodisruption. Here, the average pore size in the ILM was measured to be 69 ± 16 µm, while the average space of intact ILM between the pores was 51 ± 16 µm. This design, which creates pores for the RGCs to migrate through yet leaves large areas of ILM intact, might be the ideal manner to boost RGC integration.

In conclusion, our findings demonstrate that ICG-mediated ILM photoporation is a promising method to disrupt the ILM in a highly tunable and controlled manner. Preliminary experiments indicate that ILM photodisruption can increase donor RGC integration into the retina. Future studies will involve exploration of the impact of different laser scanning patterns on RGC transplantation.

P429

Our initial experience with intraputaminal delivery of Upstaza using MRI-guidance for cannula placement and confirmation of infusion target coverage

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Intraparenchymal (IP) infusion refers to the administration of a therapeutic directly to the brain and represents a groundbreaking approach to targeting neurological disorders at their source. One of the advantages of using the IP route of administration is that it bypasses the blood brain barrier (BBB) entirely, which can be a significant issue with therapeutics developed for systemic delivery. Additionally, IP delivery is local and therefore has less off-target side effects as seen with systemic delivery. Eladocagene exuparvovec (Upstaza™) is the first gene therapy commercially approved in the EU (July 2022) to be delivered directly to the brain for the treatment of aromatic L-amino decarboxylase (AADC) deficiency in individuals ≥18 months. This case report details the experience of our first successful MRI-guided, intraparenchymal infusion of Upstaza™ delivered directly into the human brain and was performed at Policlinico Umberto I. The patient treated is a 3 year old pediatric diagnosed with AADC deficiency. Four trajectories (two per hemisphere) were planned to target the anterior and posterior aspects of each putamen. The surgical workflow consisted of using an MRI-guided stereotactic platform (ClearPoint® Neuro, Inc.) to accurately place the cannula tip in the area of interest. The infusion was then administered with an infusion pump and a cannula containing a stepped-tip design (SmartFlow MR Compatible Ventricular Cannula®) to enable convection enhanced delivery (CED). MRI-guidance was utilized to guide the precise placement of the cannula tip to the desired target, but MRI can also be used to intra-procedurally monitor the infusion in real-time to ensure proper target coverage, if desired. Previous studies have shown that direct, intraparenchymal infusion of therapeutics for AADC deficiency resulted in improvements with cognition, communication, body weight, hypotonia, dystonia as well as a reduction of oculogyric crises. Additionally, an increase in putaminal enzymatic activity has also been noted. At this time, we are only able to confirm the safety and feasibility of intraparenchymal gene therapy infusion as no major adverse events were observed

during or following the procedure, but we are hopeful that our patient will exhibit the same level of efficacy given that the therapeutic has been delivered at such a young age. While findings to date have been encouraging, further investigations involving larger cohorts and longer-term assessments are necessary to confirm the efficacy of intraparenchymal gene therapy, optimize dose volumes and infusion rates, and to establish a new standard form of treatment for neurodegenerative disorders. Overall, this study demonstrates the safety and feasibility of direct, intraparenchymal gene therapy infusion to the brain. Furthermore, MRI-guidance can be used to confirm accurate placement of the cannula and also provide intra-procedural visualization of the infusion which can be extremely important considering the potential for reflux and perivascular off-target spread.

P430

Gene therapy for synucleinopathies: Combining vectorized antibody and miRNA-mediated lowering strategies

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Synucleinopathies are neurodegenerative disorders characterized by aggregation of alpha-synuclein protein (α-Syn), encoded by the SNCA (Synuclein Alpha) gene. Alpha-synuclein is expressed in the brain and peripherally in red blood cells. In the diseased brain, α-Syn can form toxic aggregates, which might spread in a prion-like manner as the disease progresses. Currently, there are no disease modifying therapies available for synucleinopathies. Two different therapeutic strategies are proposed to potentially halt these diseases: First, to lower α-Syn protein levels in relevant brain regions to reduce aggregation and second, to facilitate the clearance of α-Syn aggregates outside of the cell to reduce the spread of the pathology in the brain. Both strategies are the focus of many ongoing preclinical and clinical studies using different therapeutic modalities. However, delivery of these therapeutic agents to the relevant brain regions remains a challenge and repeated administration is often required. Moreover, because of the abundant presence of a-Syn in blood, peripheral administration of some of these therapeutic agents decreases their CNS bioavailability and increases the risks of anti-drug antibody formation.

We are developing two complementary AAV-based gene therapy approaches targeting α-Syn pathology. First, an engineered microRNA-based approach, using our miQURE® platform, to lower SNCA mRNA and α-Syn protein levels. We have previously shown proof-of-concept of our SNCA-targeting miQURE approach in relevant preclinical Parkinson Disease models. Second, our AbQURE™ platform technology for expression and secretion of vectorized, α-Syn targeting antibodies. In the current study we show that two different antibodies targeting the C-terminal part of α-Syn were successfully expressed in our AbQURE™ platform (AbQURE-1 and AbQURE-2) in *in vitro* models. Additionally, we obtained *in vivo* proof of mechanism by safely, efficiently, and dose-dependently expressing α-Syn-targeting antibodies in relevant brain regions, after a single direct brain administration of these vectorized α-Syn antibodies. Finally, we combined the miQURE® and AbQURE™ approaches in our GoQURE™ platform, in which we express a SNCA-targeting engineered microRNA and an α-Syn-targeting antibody from the same construct. Here, we have established *in vitro* GoQURE™ proof-of-concept for synucleinopathies. Functional antibodies were expressed in HEK293T and SH-SY5Y cells after transfection with plasmids carrying GoQURE™ constructs (GoQURE-1 and GoQURE-2). Results show that 96% of the

expressed antibodies are efficiently secreted into the medium in both *in vitro* models. Moreover, the SNCA-targeting miQURE® encoded in the GoQURE™ constructs is expressed simultaneously as the vectorized antibody.

The current study presents *in vitro* and *in vivo* proof-of-mechanism for the AbQURE™ and GoQURE™ platforms, to reduce intracellular α-Syn expression and extracellular α-Syn aggregates and ultimately decrease α-Syn toxicity in synucleinopathies.

miQURE is a registered trademark in the US and other jurisdictions; AbQURE and GoQURE are trademarks registered in the European Union and United Kingdom and pending in other jurisdictions.

P431

Synthetic neuronal promoters that surpass synapsin in the central nervous system

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The promoter is an essential *cis*-regulatory element in any DNA-based gene therapy. It directly controls gene transcription and thereby therapeutic protein expression. In the context of genetic medicines, stronger promoter activity may enable a lower vector dosage to achieve therapeutic effect, reducing safety risks associated with high vector dosages, as well as reducing manufacturing costs. In addition to strength, a tissue-specific promoter may further attenuate off-target effects and improve safety outcomes. Two well-known neuron-specific promoters are the human synapsin (hSyn) and the neuron-specific enolase (hNSE) promoters. We rationally engineered hSyn- and hNSE-based promoters to increase potency while maintaining specificity, thereby creating an improved set of strong, durable, and neuron-specific promoters amenable to central nervous system (CNS) gene therapy applications. The size of engineered promoters ranged from 1120 to 1460 base pairs. Potency and specificity were assessed *in vitro* and *in vivo*. hSyn and hNSE promoter variants were up to 7.5-fold stronger in transfected N2a cells with minimal loss of cell specificity as assessed by comparing potencies in N2a versus in HEK293T cells. The top promoter candidate had an 8-fold improvement over the parental hSyn in AAV9-transduced primary cortical neurons. Promoter cell-type specificity and strength was quantified in the mouse central nervous system after AAV9 delivery into the cisterna magna. Our top promoter was more potent *in vivo* than hSyn as well as CAG, a benchmark constitutive promoter. Together our data identified synthetic promoters with greatly improved potency for driving therapeutic gene expression in the CNS.

P432

Automatic segmentation of nonhuman primate brain structures using 3D U-net

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Nonhuman primates (NHPs) serve as robust and effective *in vivo* models in the development of novel therapeutic drugs. For procedures such as convection-enhanced delivery (CED) for intraparenchymal infusions of therapeutics to the brain, precise and accurate identification and quantification of target structures is imperative. While modern MRI-guided minimally invasive procedures facilitate highly accurate targeting, automatic targeting based on structural segmentation of various anatomical regions can further enhance the efficiency and consistency of targeting, drug delivery accuracy, and post-infusion analysis. This work introduces a machine learning-based approach for the automatic segmentation of brain structures in NHPs using optimized MRI scans. Specifically, we trained a 3D U-Net using improved MP2RAGE scans (N=15 cynomolgus macaques) to segment the putamen, caudate, thalamus, and cerebellum. Each scan underwent a 20-fold augmentation via random rotation, resulting in a total of 300 training cases. During image preprocessing, the skull was stripped from each scan and during postprocessing, only the largest segment in each label class was retained. Performance assessment was conducted on 2 test cases using the Dice Coefficient. Results showed the robustness of the proposed approach, as evidenced by volumetric visualization and quantitative assessment. Specifically, the average Dice Coefficient for the left putamen, left caudate and the entire cerebellum were 0.81, 0.81, and 0.85, respectively. This work confirms the feasibility of fully-automatic segmentation of NHP MRI data, indicating its potential to benefit minimally invasive procedures for drug delivery, such as MRI-guided CED.

P433

Pivotal safety studies for AMT-260, a novel AAV9-dual microRNA-based vector targeting GRIK2 for the treatment of temporal lobe epilepsy

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Temporal Lobe Epilepsy (TLE) is a type of focal epilepsy characterized by recurrent seizures generated in the hippocampus. Mossy fiber sprouting from dentate granule cells (DGCs) is a consistent anatomical hallmark of TLE, leading to synaptic reorganization in an aberrant excitatory network. In TLE, the DGCs operate via aberrant ectopic expression of GluK2/GluK5 kainate type receptors which plays a central role in the generation of seizure activity. AMT-260, an AAV9 vector coding for 2 microRNAs was designed to knockdown GluK2 expression by specifically targeting GRIK2 mRNA.

AMT-260 administered bilaterally into the hippocampus of a mouse model for TLE (pilocarpine) has already demonstrated efficacy in seizure reductions and improvement in general animal health. To further assess the safety of AMT-260 to prepare for entry into clinical trials, studies in non-human primates (NHP) were conducted over 1, 3 and 6 months duration. Administration to the hippocampus using MRI-guided convection-enhanced delivery with a ClearPoint® cannula, anticipating the proposed clinical route, was performed bilaterally. AMT-260 was well tolerated and without adverse findings at the predicted clinical dose. Cell transduction by AMT-260 was restricted to the CNS with no peripheral exposure. microRNA expression was restricted to neurons and focally expressed in the hippocampus and adjacent entorhinal cortex. microRNA expression was strongly correlated with GRIK2 mRNA knockdown, achieving more than 95% reduction at the highest doses. Safety and vector pharmacokinetics data from mouse and NHP studies show a promising safety profile for further clinical development.

P434

Nose-to-brain delivery of siRNA-loaded nanoparticles leads to downregulation of alpha-synuclein in a Parkinson`s disease mouse model

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Potential strategies to develop new treatments for Parkinson`s disease (PD) target specific proteins and signaling pathways associated with PD. One such protein is alpha-synuclein (aSyn), which accumulates in neurons of PD patients and contributes to the degeneration of these cells. A promising new approach to lower the protein level of aSyn is the therapeutic use of small interfering RNAs (siRNAs). Efficient delivery of siRNA is challenging due to the instability of the RNA and the need to overcome or circumvent the blood-brain barrier. Currently, delivery is largely limited to injections into the cerebrospinal fluid, an invasive surgical procedure. To overcome those challenges, we developed a nanoparticle-based approach for intranasal delivery of small RNAs, opening this gene therapy strategy to broad clinical application.

In this project, we worked with seven different polymeric nanoparticles (NPs), which were tested *in vitro* for their ability to penetrate neuronal cells. Differentiated SH-SY5Y neuroblastoma cells were incubated with fluorescent-labeled control siRNA-NPs and evaluated by confocal microscopy. To characterize NPs further and to check which ones are most promising to deliver siRNAs from the nose to the brain, we administered different NPs loaded with Alexa Fluor (AF) 647-labeled control siRNA intranasally to aSyn overexpressing (Thy1-aSyn) mice once daily on four consecutive days. For functional experiments, two-month-old Thy1-aSyn mice received either nanoparticles loaded with siRNA targeting human *SNCA* mRNA (siSNCA-NPs) or control siRNA (siCtrl)-NPs intranasally (4 µg/day on four consecutive days). In both experiments, mice were sacrificed 24 hours after the last application and brains were collected for immunohistochemical as well as western blot analysis.

All tested NPs were able to penetrate SH-SY5Y neuroblastoma cells. Furthermore, all AF647-labeled NPs reached the brain after intranasal application. They distributed extensively across the brain parenchyma and were detectable in several different regions including the

olfactory bulb, substantia nigra and prefrontal cortex. A quantitative evaluation revealed that cationic polymers reached the brain in the highest amount. Mice did not show overt adverse behavioral effects nor increased reactivated microglia. After only four days of intranasal administration, siSNCA complexed with polymeric NPs reduced brain aSyn levels. Efficacies depended on the NP, with up to 70% *SNCA* knockdown compared to siCtrl-NPs in Thy1-aSyn mice.

In conclusion, non-invasive NP-mediated intranasal application of siSNCA to aSyn overexpressing mice is able to reduce aSyn levels in the brain and could be a novel therapeutic approach to treat PD.

P435

The microRNA-responsive autoregulatory element from TSHA-102 for Rett Syndrome modulates therapeutic transgene expression in response to cellular MeCP2 in mouse and human cell lines

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Rett Syndrome is a rare, severe neurodevelopmental disorder caused by mutations in the *MECP2* gene, encoding the highly conserved transcriptional regulator methyl-CpG binding protein 2 (MeCP2). Intracellular levels of MeCP2 are tightly regulated to ensure proper function. Pathological phenotypes are observed for mutations that result in either over- or under-expression, which manifest in similar clinical symptoms.

We have developed an investigational AAV9 gene therapy, TSHA-102, which encodes miniMeCP2 (a miniaturized version of the human MeCP2) under control of a unique miRNA-Responsive Auto-Regulatory Element (miRARE) in the transgene's 3' untranslated region (UTR). The miRARE contains binding sites for several CNS-expressed microRNAs (miRNAs) whose cellular levels correlate with endogenous MeCP2, which may establish a negative feedback loop that could prevent toxic overexpression of the transgene. TSHA-102 appears to improve overall survival, weight gain, and motor and other phenotypic abnormalities in *Mecp2* $^{-/y}$ male mice (*Mecp2* KO), while showing no signs of toxicity in *Mecp2* wild-type mice, rats, or non-human primates. To date, the post-transcriptional regulation of mini*MECP2* by the miRARE has not been explored in an experimentally tractable cell culture system.

Here, we investigated miniMeCP2 expression and miRARE function in immortalized human (2v6.11) and neuronal mouse cells (N2a; an in vitro model for the CNS cells) targeted with our gene therapy. In TSHA-102-transduced 2v6.11 cells (derived from a HEK293 cell line), miniMECP2 mRNA and protein expression was linear over a multiplicity of infection (MOI) range of 1 – 20 x 10^5, with no observable impact on cell viability. Similar trends of mRNA and protein expression profiles were observed with plasmid transfection.

To test whether the presence of miRARE in TSHA-102 modulated miniMeCP2 expression in immortalized cells, we transfected both N2a and 2v6.11 cells with expression plasmids containing mini*MECP2* plus either the miRARE (pTSHA102^{miRARE}) or a control 3'UTR element (pTSHA102^{SV40}). Inclusion of the miRARE was associated with minimal (N2a) or no (2v6.11) effects on transgene

mRNA expression, but with notable effects on miniMeCP2 protein levels in both cell lines. Thus, relative to pTSHA102^{SV40}, miniMeCP2 protein expression from pTSHA102^{miRARE} was reduced 31%-40% (N2a) and 20-46% (2v6.11), depending on MOI, suggesting a greater effect of the miRARE on translation than on RNA stability.

To assess whether miRARE-mediated silencing responded to cellular MeCP2 function, we used CRISPR to create MeCP2^{null} (biallelic mutation) and MeCP2^{low} (monoallelic mutation) 2v6.11 subclones. Following transient transfection with pTSHA102^{miRARE}, miniMeCP2 expression was modestly (23%) and dramatically (340%) increased in MeCP2^{low} and MeCP2^{null} cells, respectively, relative to parental (MeCP2^{WT}) 2v6.11 cells.

Together, these findings may suggest that miRARE post-transcriptionally modulates transgene-expressed miniMeCP2 levels in immortalized mouse and human cells. As hypothesized, miRARE-mediated modulation of miniMeCP2 expression appears to require cellular MeCP2 function. Further experiments are ongoing to characterize miRARE function and explore the relevance of cellular MeCP2 background for transgene expression in this system.

P436

Evaluation of human neuroblastoma cell lines based *in vitro* potency assays for a neuropeptide expressing AAV1 gene therapy

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Drug-resistant focal epilepsy is a common, chronic, debilitating neurological disease affecting up to 80% of patients with mesial temporal lobe epilepsy. In recent years, AAV gene therapy gained increasing interest as potential novel treatment strategy for focal epilepsies. The possibility to directly inject the therapeutic AAV vector into the focus allows for a targeted treatment. Neuropeptides have been put into the focus in relation to focal epilepsy due to their modulatory effect on neuronal excitability. Compared to systemic drug delivery, direct injection and expression of neuropeptides from a therapeutic vector allows long-term focal peptide delivery and reduction of off-target effects. When developing an AAV gene therapy, determining vector potency is a critical attribute. The assay requires to be transgene-specific and replicate the biological mechanism of action as closely as possible. For a long time, *in vivo* models were relied on to test and determine vector quality. However, with a global effort to replace, reduce, refine animal testing, as well as regulatory requirements to develop and implement *in vitro* models, the need to evaluate the correct model is pivotal. This can be difficult for complex diseases like epilepsy. Additionally, the lack of well characterized human cell lines for cell or tissue types of choice makes it challenging to develop a potency assay. It is therefore important to carefully choose cell lines based on parameters such as transduction efficiency for the AAV serotype of choice, and proper expression and processing of transgene products. We evaluated two human neuroblastoma cell lines for their suitability towards neuropeptide expressing AAV1 vectors. We specifically looked at the cell lines transducibility, single-stranded and self-complementary AAV expression, mRNA kinetics, as well as neuropeptide expression and processing to mature peptides over a wide range of multiplicities of infections. We came up with an all-in-one biopotency assay that allows for parallel determination of fully processed neuropeptide expression, RNA and DNA from a single-infected well. Finally, we validated the newly developed *in vitro* potency assay

analysing several dynorphin-expressing AAV vectors in parallel to an established *in vivo* assay. Expression levels of fully-processed, mature dynorphin B in the cell-based assay correlated with expression levels in vector-transduced mouse brain tissue. The novel human neuronal cell-based *in vitro* assay will shorten vector screening, allow for AAV batch to batch validation, and reduces the need for animals in potency testing.

P437

Surface augmented mesenchymal stem cell-derived extracellular vesicles promote optic nerve recovery by retinal ganglion cell and macrophage modulations revealed by single-cell RNA sequencing

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Extracellular vesicles (EVs) derived from mesenchymal stem cells have shown promise as a potential therapeutic approach for optic nerve injury. However, further investigation is needed to enhance their effectiveness. This study aimed to investigate the impact of functionally enhanced EVs derived from human placenta derived mesenchymal stem cells on retinal ganglion cells and macrophages in optic nerve injury. To improve targeting and recovery function, the EVs were conjugated with the c(RGDyK) peptide (c(RGDyK)_EVs), and their treatment effects were analysed. In vitro, R28 cells, retinal precursor cells, were subjected to CoCl2-induced injury and treated with c(RGDyK)_EVs for 24 hours. To elucidate the regulatory mechanisms underlying EV-mediated recovery in optic nerve disease models, immunoblot assays and single-cell RNA sequencing were performed using both in vitro and in vivo optic nerve disease animal models, as well as retina organoids. Our findings demonstrated that EVs effectively restored the abnormal regulation of neuroregeneration markers in optic nerve injury models. Notably, the functionally optimized c(RGDyK)_EVs exhibited superior targeting capabilities and modulated neuroregeneration in cases of hypoxic damage and inflammation. Single-cell RNA sequencing analysis of R28 cells revealed significant regulation of gene transcription involved in retinal ganglion cell (RGC) regeneration, neuronal growth, and mitochondrial homeostasis by c(RGDyK)_EVs. In summary, this study highlights the enhanced therapeutic potential of c(RGDyK)_EVs over naive EVs in the context of optic nerve disease. The findings suggest that c(RGDyK)_EVs hold promise as an alternative therapy for optic nerve injury. The comprehensive analysis using single-cell RNA sequencing provides valuable insights into the underlying molecular mechanisms involved in EV-mediated neuroregeneration.

P438

Elucidation of the neuropathological defects in iPSC-derived iNeurons from patients with myotonic dystrophy type 1

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Myotonic dystrophy type 1 (DM1) is an inherited multi-systemic disorder, originating from the expansion of an unstable CTG repeat in the 3' UTR of the *DMPK* gene. Typically, a longer repeat correlates with more severe symptoms and an earlier age of onset. The disease pathogenesis is thought to be mainly caused by a deleterious gain-of-function effect of the expanded *DMPK* transcripts. Aside from features like myotonia and muscle wasting, DM1 is also characterized by cognitive impairments such as executive dysfunction, visuospatial deficits and excessive daytime sleepiness. Studies in mouse models have shown that not only neurons are affected but also astrocytes. However, knowledge about the precise interplay between these two cell types and the underlying mechanisms that cause the cognitive defects is limited. We therefore developed a cell-based model to elucidate the neuropathological defects.

We successfully generated induced pluripotent stem cells (iPSC) from fibroblasts from four DM1 patients and differentiated these to glutamatergic excitatory neurons (iNeurons). For patient 2, we generated an isogenic line in which the expanded CTG repeat was excised by CRISPR/Cas9. To characterize these lines, we studied DM1-specific hallmarks, such as RNA foci. Additionally, we performed initial neural network measurements of iNeurons by multi-electrode array (MEA). Preliminary data suggest that DM1 neurons appear to be hyperexcitable *in vitro*, similar to what was found in a DM1 mouse model. Next, we aim to further identify deficits of patient-derived neurons and to identify disrupted biological processes underlying the DM1-specific defects. We will investigate somato-dendritic morphology by reconstruction of immunofluorescent images. An in-depth analysis of the electrophysiological MEA profiles of DM1 neurons will be performed, and identified neural network aberrations will be combined with transcriptomics (RNAseq) to identify molecular pathways that are disrupted due to *DMPK* repeat expansion.

This 2D-model will be a screening platform for different small molecules and antisense oligonucleotides (AONs) that can reverse the phenotype seen on MEA. With these, we hope for a first step towards a treatment of the CNS symptoms experienced by DM1 patients.

P439

Local intracerebral injections of a recombinant adeno-associated virus for the treatment of Lafora disease.

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Lafora disease is a rare and fatal form of progressive myoclonic epilepsy caused by mutations in two genes, *EPM2A*, which encodes laforin, a dual specificity phosphatase or *EPM2B*, encoding an E3 ubiquitin ligase known as malin. Both interact forming a functional complex that is involved in the regulation of glycogen metabolism, proteasome-mediated degradation, and autophagy. Mice deficient for laforin (*Epm2a*$^{-/-}$) or malin (*Epm2b*$^{-/-}$) develop most of the disease characteristics, including cognitive and motor impairment, neurodegeneration, neuroinflammation, presence of Lafora bodies, and epileptic activity. Restoring the activity of laforin or malin by replacing the defective gene using recombinant adeno-associated virus (rAAV) might be a promising therapy. Previous studies from our laboratory have shown beneficial outcomes of a single intracerebroventricular injection (ICV) of a rAAV vector carrying the human *EPM2A* gene (rAAv2/9-CAG-h*EPM2A*) in the *Epm2a*$^{-/-}$ mouse model of Lafora disease. Laforin immunostaining showed that the distribution of the transgene was mostly limited to the hippocampus and the area around the lateral ventricles. Considering the general neurodegeneration, neuroinflammation, and neuronal excitation/inhibition imbalance throughout the brain, widespread transgene expression might be necessary to increase the efficacy of the vector. In this work, we performed intracerebral injections (IC) of rAAV2/9-CAG-h*EPM2A* into seven brain regions of *Epm2a*$^{-/-}$ mice, bilaterally in the prefrontal cortex, caudate-putamen and lateral ventricles, and one single injection in the cerebellum. GFP immunofluorescence showed that IC administration of the vector achieves a wider biodistribution. GFP-positive cells were found in the prefrontal cortex, hippocampus, caudate-putamen, cerebellum, medulla, and regions surrounding the lateral ventricles. Given the promising results with a single ICV injection, we expect to observe greater histopathological and neurological improvements in the IC treated mice.

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PromPT® - Promoter Precision Technology, a platform for design, screening and characterisation of cell type specific promoters

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1: AskBio Europe

Gene therapy requires expression cassettes which are both highly selective and adjustable. To address this, we developed PromPT®, a bioinformatics platform for the identification, characterisation, and design of cis regulatory elements (CREs) for synthetic promoters. In this study, we showcase a practical application of PromPT® for promoter design and evaluation of CREs for widespread expression in the brain.

AAV9 based libraries were created to screen 228 CREs and 20 synthetic promoters predicted to be active in the brain. We constructed tiling library using all 228 CREs as the foundation. Analysing the signal from the tiling library, we identified active regions within the candidate CREs. We screened 60 out of the 228 candidate CREs in combination with the 20 synthetic promoters using a barcoded reporter assay in vivo. Tissue sections were assayed using the 10x genomics Visium platform, and promising candidates were individually validated using fluorescence microscopy.

We believe, the combination of these library approaches provides a robust platform for identifying and characterizing CREs, thereby expediting the synthetic promoter design cycle.

P441

Direct Intra-Cerebellar Delivery and Infusion Coverage of AAV5-GFP Using Convection-Enhanced Delivery in Nonhuman Primates

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Many neurological diseases involve the cerebellum, and can manifest clinically as ataxia, hypotonia, and nystagmus. Here, we present data compiled from 2 recent studies conducted in nonhuman primates (NHPs) that received AAV5-GFP into the cerebellum. Using convection-enhanced delivery (CED), all animals received a single, unilateral infusion and these data demonstrate the feasibility and tolerability in targeting the cerebellum in NHPs. Animals were monitored daily for signs of neurological impairment and weight loss until necropsy, which occurred 4-6 weeks post infusion. Histological and immunohistochemical analyses were used to determine tissue response to cannula insertion and presence of the GFP transgene. No untoward side effects were noted post infusion. As important, we denote a trans-parietal surgical trajectory that passes through the overlying neocortex and tentorium to reach the cerebellum. The practicality and usefulness of performing these procedures under intraprocedural MRI-guidance allowed us to examine vector transport and infusion patterns unique to this anatomical region, and accurately measure infusion coverage to correlate volume of distribution to volume of infusion – a key consideration for clinical translation. In addition, as part of this presentation, we highlight novel methodological improvements for targeting the cerebellum without passing through the neocortex or tentorium with trans-suboccipital surgical trajectories. Taken together, these data provide considerations for direct targeting of the cerebellum in NHPs to focally deliver a gene therapy product that could incorporate another gene-of-interest such as frataxin, which is deficient in Friedreich Ataxia. Therefore, these studies provide translatable data that could be used in relevant clinical conditions with a cerebellar component.

P443

The guardians of balance: mesenchymal stem cells as a promising strategy to restore blood-brain barrier integrity in spinocerebellar ataxia type 3

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Spinocerebellar Ataxia type 3 (SCA3), or Machado-Joseph disease, is a neurodegenerative disorder caused by a CAG expansion in the MJD1/ATXN3 gene. We recently found evidence of disease-associated breakdown of the blood-brain barrier (BBB), an important cellular barrier that maintains the central nervous system's homeostasis. Furthermore, we previously demonstrated that MSC administration can ameliorate the phenotype of SCA3 transgenic mice. However, the impact of MSCs on BBB integrity in SCA3 remains unexplored.

In this study, we aimed to: 1) further elucidate the molecular mechanisms involved in BBB dysfunction in SCA3; and 2) evaluate the therapeutic potential of MSCs in mitigating BBB disruption.

Our results revealed significant dysregulations in the neurovascular unit in SCA3 mouse models, including increased BBB permeability and angiogenesis, and alterations in blood flow. These changes were preceded by neuroinflammation and the dysregulations of expression levels/subcellular localization of key adherent and tight junction-associated proteins, including Claudin-5, Occludin, Ve-Cadherin, and the cytoplasmic adaptor zonula occludens (ZO)-1.

Furthermore, we also detected that the administration of mesenchymal stem cells (MSC) may partially restore BBB impairment in SCA3 mice. Indeed, treatment with two consecutive intravenous injections (one week apart) of MSC resulted in improved gait/motor performance and concurrently attenuated vascular dysfunction. Specifically, MSC-treated mice exhibited a decrease in collagen IV surface area, regulated blood flow, and exhibited a tendency to diminish BBB permeability. So far, we have also detected MSC modulation of Ve-cadherin cleavage and observed a decrease in astrogliosis in treated animals.

In conclusion, our findings suggest that increased BBB permeability observed in SCA3 may be associated with dysregulation of TJ/AJ proteins and neuroinflammation. Moreover, MSC administration partially reverted BBB impairment, providing valuable insights into potential therapeutic strategies for SCA3.

P444

Enhancing differentiation of adipose-derived mesenchymal cells for retinal pigment epithelium repair

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Adipose-derived mesenchymal cells (AD-MSCs) hold great promise in the field of cell therapy and regenerative research, thanks to their ability to differentiate into various cell types and facilitate the repair of damaged cells. In the case of retinal diseases such as AMD, the retinal pigment epithelial cell layer (RPE) is damaged in the late stages of the disease, necessitating the replacement of these damaged cells.Human AD-MSCs were cultivated under standard conditions, with 5% CO2 at 37°C in a humidified cell culture incubator. Low glucose DMEM medium supplemented with GlutaMAX™, 10% FBS, and 2% antibiotics (100 U/ml penicillin and 100 µg/ml streptomycin) was used. The cells were then incubated with RPE condition medium (RCM) and treated with different drugs (VIP, NIC, ATRA) and their combinations in 6-well culture plates for 15 days. The impact of these culture conditions on AD-MSC differentiation into RPE was assessed through Western blot technique and immunofluorescence microscopy, quantitatively measuring the expression levels of RPE-related proteins: RPE65, Bestrophin, PCK, Ck8/18. Statistical analysis was conducted to identify significant differences in the impact of culture conditions on hAD-MSC differentiation into RPE. The results demonstrated a significant increase in the expression levels of RPE65, Bestrophin, PCK, and Ck8/18 in hAD-MSCs cultivated under RCM culture conditions compared to the control group. Further data comparisons revealed that the culture conditions containing drug combinations exhibited significantly higher expression of RPE65, Bestrophin, PCK, and Ck8/18 proteins, surpassing the other conditions. The presence of drug combinations in the culture conditions proved to be more effective in stimulating the differentiation of hAD-MSCs into RPE cells. These findings offer valuable insights for optimizing the use of AD-MSCs in the repair and regeneration of retinal tissues affected by diseases like AMD.

P445

Novel Cassette Design Technology for optimized Protein Splicing reconstitution of large proteins

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Protein Splicing is a post-translational process catalyzed by a family of proteins known as inteins. During this process, an intein domain catalyzes its own excision from a larger precursor protein and simultaneously ligates the two flanking polypeptide sequences (exteins) together. Protein Splicing has been efficiently used for the reconstitution of large genes from two smaller gene fragments using dual AAV vectors. This strategy is based on splitting the target gene into two fragments and fusing each of them to the coding sequences of two complementary split-inteins. Upon infection of the target cells the two transgenes are transcribed and translated and at the protein level the split inteins will carry out the Protein Splicing reaction to generate the desired target protein. One particularity of the Protein Splicing approach is that two protein fragment starting materials are produced in the transduced cell, which need to react to form the full-length product. Also, after splicing the split-inteins are excised as side-products of the process. For

certain proteins or therapeutic indications, it might be desirable to minimize the formation of these side-products, while maximizing the formation of the desired full-length target protein. To address this question, we have developed a proprietary cassette design technology based on the combination of proprietary engineered split inteins with degradation signals to prevent the accumulation of both protein fragment starting materials and excised inteins. We have validated this novel approach using EGFP as a model protein, and also the therapeutic protein ABCA4 in vivo in a mouse model of Stargardt disease, demonstrating that the technology results in the formation of the desired product (ABCA4) in the absence of ABCA4 fragments or excised inteins.

P446

Definitive results of the preclinical study conducted in rodents to determine the neuroprotective and neurotrophic effects of the autologous fat-derived stromal vascular fraction (SVF) in the acute management of spinal cord contusions

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Spinal cord injuries (SCI) lead to functional alteration with important consequences such as motor and sensory disorders. The repair strategies developed to date remain ineffective. The adipose tissue-derived stromal vascular fraction (SVF) is composed of a "cocktail" of mesenchymal and hematopoietic stem cells with trophic, pro-angiogenic and immunomodulatory effects. Numerous therapeutic benefits were shown for tissue reconstitution, peripheral neuropathy and for the improvement of neurodegenerative diseases. Our strategy is based on an autologous injection of the SVF within 4 hours after SCI. To check our hypothesis, we conducted a pre-clinical study in adult male rats. Contusions performed at thoracic level T10 using an impactor, all the animals were paraplegic. The epididymal fat removed in a second time, then the autologous SVF is purified (>90% of viability), before 1 million of cells are directly injected into the lesion. Autologous SVF implantation promotes 1) locomotor recovery (BBB test, Ladder rung walking test, Catwalk), 2) H-reflex normalization, and ventilatory frequency adjustment to an isometric exercise. 3) *In vivo* 7T MRI, shows signs of regeneration and revascularization. We also identified a biomarker for the following of the inflammation. These results were confirmed by 4) studying proinfammatory cytokines (IL-1, IL-6, TNF-α) by ELISA, and 5) immunohistological analyzes clearly showed signs of revascularization and regeneration within the spinal cord lesions (angiogenesis markers with CD31, neuronal markers with MAP2 and axonal regeneration with GAP43). 6) Finally, intensive animal training significantly potentiates the regenerative effects of the autologous SVF. In conclusion, these encouraging results demonstrate significative recovery in rodent post-SCI after implantation of autologoue SVF cells that could be improved by treadmill exercise. The work is currently being carried out in pigs before moving on to humans.

P447

Elevating tRNA^{Gly} levels as a therapeutic approach for glycyl-tRNA synthetase associated peripheral neuropathy

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Charcot Marie Tooth (CMT) disease is the most frequent inherited neuromuscular disorder (1 in 2500), with around 300,000 patients in Europe alone, and 3.2 million people worldwide. CMT leads to selective degeneration of peripheral motor and sensory nerves, leading to progressive muscle weakness and wasting, and a decrease or loss of sensation. Symptoms slowly progress over decades, with patients needing walking aids and ultimately often becoming wheelchair bound. Despite these debilitating features, CMT patients have a normal life span. Thus, life-long symptomatic treatment is required. However, there is no FDA- or EMA-approved therapies.

Dominant heterozygous mutations in seven aminoacyl-tRNA synthetases (aaRSs) have been causally linked to CMT, making aaRSs the largest protein family implicated in CMT. aaRSs ligate amino acids to their corresponding tRNA, which is the first step in protein synthesis. Our lab recently showed that the glycyl-tRNA synthetase with CMT-associated mutations (GlyRS-CMT) sequesters tRNA^{Gly}. Depletion of the free cellular tRNA^{Gly} pool elicits ribosome stalling on glycine codons, activation of the integrated stress response and decreased protein synthesis. These molecular hallmarks and the motor performance defects of CMT-GlyRS mouse models are rescued by transgenic tRNA^{Gly} overexpression. High levels tRNA^{Gly} overexpression was achieved by integrating several copies of tRNA^{Gly} genes in the mouse genome.

Here, we aim to test the therapeutic potential of elevating tRNA^{Gly} levels by adeno-associated viral vector (AAV9)-mediated gene delivery. First, we are developing and testing tRNA^{Gly} constructs that can be packaged in AAV9 vectors and induce high levels of tRNA^{Gly} overexpression in motor neurons. Then, we want to asses if this strategy can be used to prevent, stop, or even revert the progression of the disease in mouse models of CMT-GlyRS.

P448

AAV gene replacement therapy for *RPE65*-mediated inherited retinal dystrophies

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Inherited retinal dystrophies (IRD) are a heterogeneous group of diseases affecting more than 2 million people worldwide. Caused by mutations in almost 300 distinct genes, IRDs result in severe visual impairment due to dysfunction and degeneration of the photoreceptors, retinal pigment epithelium (RPE), or the choroid. Advances in DNA delivery to the retina has led to a new era of research into gene therapy for these vision-threatening disorders. Gene therapy is a compelling approach due to the monogenic nature of most IRDs, with the retina being a favorable target for administering genetic vectors due to its immune-privileged environment, direct visibility, and

multiple methods to assess sensitivity and function. It is estimated that ~6,660 and ~8,200 individuals living with IRD associated with the *RPE65* gene mutations in the United States (US) and Europe, respectively. The *RPE65* gene plays a critical role in the visual cycle by producing the all-trans retinyl ester isomerase enzyme. Although Luxturna, an adeno-associated virus serotype 2 (AAV2) vector carrying the normal functional *RPE65* gene, became the first US FDA-approved gene replacement therapy in 2017, data from the literature have suggested that the transduction efficiency of RPE cells with AAV2 is worse than that of other serotypes. Therefore, Luxturna required higher total vector dose and larger volume as 1.5 $x10^{11}$ vg /300uL per eye to circumvent the lack of efficient AAV vectors. Generally, lowering the total vector dose and total volume injecting into the retina is considered to be beneficial, as higher doses and larger volume have been associated with safety concerns. Here, we developed HG004, a non-AAV2 gene replacement therapy product carrying human *RPE65* gene. In a head-to-head animal comparative study with the same dose of AAV2-*hRPE65* and HG004, HG004 enabled high expression of hRPE65 in the eyes of *Rpe65*$^{-/-}$ mice and restored extinguished electroretinogram (ERG) signals while AAV2-*hRPE65* would need to increase 10-fold in order to achieve the same level of efficacy as HG004. The efficacy of HG004 was dose-dependent with the optimal dose leading to a 70% restoration of retinal function such as the scotopic ERG amplitude values in *Rpe65*$^{-/-}$ mice when compared to wildtype mice. Additionally, HG004 restored and protected the structural integrity of the retina and significantly delayed retinal degeneration. Furthermore, preclinical safety studies in non-human primates revealed that HG004 is mainly distributed within the retina and is well-tolerated at different doses without any systemic toxicity related to HG004. Based on these comprehensive preclinical data, HG004 was granted orphan drug designation (ODD) by the US FDA and received both IND approval by the US FDA and China Center for Drug Evaluation (CDE) early this year. Recently, we have already dosed patients in our investigator-initiated trial in China demonstrating human proof-of-concept of HG004. Currently, our preliminary clinical data demonstrates a substantial restoration of vision that was progressing toward complete blindness even the starting dose level of HG004 is only 1/25 of Luxturna in Phase 3 clinical trial. Notably, neither adult nor pediatric patients receiving HG004 experienced serious adverse events or retinal detachments.

P451

Adoptive immunotherapy with pentavalent-specific T cells for the treatment of opportunistic infections post haploidentical transplantation

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Adoptive immunotherapy with pathogen-specific T cells (pSTs) offers an attractive alternative for the management of viral and fungal infections, representing one of the deadliest complications of allogeneic hematopoietic cell transplantation. We here present the results of an on-going phase I/II study, evaluating the safety and efficacy of donor-derived pentavalent-specific T cells (penta-STs) simultaneously targeting cytomegalovirus-CMV, Epstein Barr virus-EBV, BK virus-BKV, adenovirus-AdV and *Aspergillus fumigatus*-AF (*EudraCT2020-004725-23*, *NCT05471661*). Penta-STs were generated after pulsing peripheral blood mononuclear cells from

immunocompetent donors with peptides spanning immunogenic EBV *(LMP2*, *EBNA1*, *BZLF1)*, Adv *(Hexon*, *Penton)*, CMV *(pp65*, *IE1)*, BKV *(Large T*, *VP1)* and AF *(Gel1*, *SHMT*, *Crf1)* antigens followed by a 10-day culture with IL-4/IL-7. The specificity of cell products and the circulating specific T-cells were assessed by Elispot. Penta-STs are infused at $2x10^7$cells/m^2/dose in patients transplanted from haploidentical donors who develop post-transplant an infection from the targeted pathogens while they receive 0-0.5mg/kg methylprednisolone at the time of infusion. Patients' responses to penta-STs and subsequent reactivations are recorded up to 6 months post-transplant. To date, we have generated 16 GMP-grade products reaching a ~4.7 fold expansion over baseline and an average of 173±22x10^6cells. Penta-STs were enriched in $CD3^+$cells (91±1%), comprised of $CD4^+$(57±4%) and $CD8^+$ cells (34±4%) and expressed central (54±5%) and effector memory markers (27±5%). All products were specific against the pathogens for which the donor was seropositive; 11/16 presented activity against CMV [1,301±236 spot forming cells (SFC)/2x10^5], 16/16 against EBV (915±155 SFC/2x10^5), 16/16 against Adv (1131±183 SFC/2x10^5), 16/16 against BKV (557±138 SFC/2x10^5) and 11/16 against AF (182±42 SFC/2x10^5). To date, seven patients received penta-STs for single (EBV n=1, CMV n=2), double (CMV+EBV n=1; EBV+BKV n=2) and triple viral infection (CMV+EBV+BKV n=1) post-transplant. No patient developed AdV or AF infection so far. Penta-ST infusion was safe, with no infusion-related toxicity, aGVHD occurrence or cGvHD exacerbation attributed to the cells. From a total of 12 infections (including 2 EBV diseases), 11 complete responses occurred (EBV n=4, CMV n=4, BKV n=3) and 1 partial response (EBV). The clinical response correlated with expansion of the circulating respective pSTs. Importantly, 3/12 infections (EBV, BKV) resolved without additional drug therapy while one patient with EBV-PTLD received only a short course (one dose) of anti-CD20 treatment due to rapid and complete resolution of lymphadenopathy post-infusion. Notably, 8 viral reactivations occurred in total [EBV n=2, CMV n=5 (seronegative donors n=2), BKV n=1)], at a median time of 6 (2-6) months post infusion, from which, 6 resolved completely and one remains stable at the end of follow-up (6mo). Notably, 2/8 viral reactivations resolved without any drug therapy and again, complete responses correlated with pathogen-specific T-cell expansion. Our data show that pST therapy is a feasible and safe option for patients at high risk for opportunistic infections after allogeneic transplantation, potentially minimizing the need for drug therapy and reducing reactivations.

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P452

Engineering chimeric antigen receptor (CAR) directed to CD84 for acute leukaemia

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Chimeric antigen receptor (CAR) T-cell therapy has obtained outstanding results in the last years. Clinical success led to the approval of several CAR T products for the treatment of patients diagnosed with B-cell malignancies with anti-CD19 CAR T cells (B-ALL) and for patients diagnosed with multiple myeloma (MM) with anti-BCMA CAR T cells. However, none has yet been approved for other haematological malignancies, such as acute myeloid leukaemia (AML) or T-cell acute lymphoblastic leukaemia (T-ALL). Here, we present the first-in-class CAR T cell directed towards CD84 (CART84) for the treatment of haematological malignancies.

CD84 (SLMAF5) is a receptor widely expressed in immune cells, and it has been reported to be overexpressed in chronic lymphoid leukaemia. The GEPIA analysis shows that CD84 mRNA expression is ten times higher in AML than in paired healthy samples. We analysed AML and T-ALL primary samples and revealed that CD84 is overexpressed on the surface of leukaemic cells by flow cytometry. On the other hand, immunohistochemistry analysis of healthy human tissues revealed that CD84 is not expressed in lung, liver, kidney, myocardium, skin, or brain (cerebral cortex and cerebellum), although expression was detected in tissue macrophages as expected.

We engineered several second-generation CART84 with a CD8a hinge and transmembrane, a 4-1BB costimulatory and CD3z signalling domain. Each CART84 construct differ in the single-chain variable fragment (scFv) domain, being murine or fully human. We generated 17 murine monoclonal antibodies (mAb) by immunizing BALB/c mice with the human CD84 protein. The fully human scFv derived from a phage-display library. Based on their affinity and domain binding, we chose 5 murine mAb (152-1D5, 153-4D9, GYCD84.1.226, GYCD84.3.89, GYCD84.1.7) and 3 fully human scFv (R3-B3, R3-G7, R3-H3). The scFv design differs in the order of their variable region of the heavy (VH) and light (VL) chain and the linker length between them, with either three (S3) or four (S4) motives (GGGGS).

The majority of CART84 exerted a high *in vitro* cytotoxicity towards cell lines from different haematological malignancies including aggressive B-cell lymphoma (Ramos), AML (MOLM-13) and T-ALL (MOLT-4). However, fully human CART84 were less cytotoxic towards some malignancies (AML and T-ALL) and showed a different cytokine profile than CART84 with murine scFv. While most CART84 displayed a high cytotoxicity towards primary leukaemic cells from AML and T-ALL, the cytotoxic effect of CART84 against CD34+ hematopoietic stem cells was much lower. In an immunodeficient NSG mouse model, CART84 controlled both MOLM-13 (AML) and MOLT-4 (T-ALL) tumour progression and increased mice survival in comparison with the control group

treated with untransduced T cells (UT). Similarly, positive results were obtained in AML and T-ALL PDX models.

CART84 displayed high cytotoxicity towards AML and T-ALL both *in vitro* and *in vivo*. Our data support the therapeutic use of CART84 for acute leukaemia. However, due to its potential myelotoxicity, we encourage it as a bridge therapy to allogeneic hematopoietic stem cell transplantation for patients with R/R AML and T-ALL.

P453

Successful *in vivo* production of functional CD19 CAR-T cells using an innovative 4th generation lentiviral vector

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While significant clinical benefit has been observed in patients receiving autologous CAR-T therapies, there are several challenges associated with this approach which limits access to therapy: cell processing is complex, slow, and costly, and CAR-T cell fitness is impacted during manufacture which can impair clinical success. Furthermore, the limited availability of appropriate GMP manufacturing facilities and clinical sites, restricts the geographical locations in which treatment can be offered.

Generation of CAR-T cells within the patient (*in vivo*) would bypass the need for *ex vivo* cell manufacture and the associated challenges offering a much more accessible treatment option for patients.

We have successfully generated functional CD19 CAR-T cells *in vivo* using several innovations based on the latest TetraVecta™ (4th generation) lentiviral vector system. The TetraVecta™ system consists of the 2KO genome™, TRiP System™, MaxPax™ and SupA-LTR™ technologies which further improve the quality, safety expression level and potency of vectors, while increasing packaging capacity. The TRiP System™ avoids CAR incorporation into the vector particle and inadvertent transduction of target tumour cells thereby masking of the antigen and loss of tumour control. Using this platform, we generated a CD8-specific vector (Ret8NIV) pseudotyped with a retargeted Nipah envelope and encoding a CD19 CAR (FMC63-41BB-CD3zeta). These vectors specifically transduced CD8+ T-cells and generated functional CD19 CAR-T cells which were able to kill MEC-1 tumour cells *in vitro*. In addition, using the TRiP System™ we were unable to detect CAR protein on the vector particles and saw no evidence of transduction of CD19+ B cell lines. When these vectors were administered into mice with a humanised immune system they were well tolerated and, importantly, produced CD8+ CAR-T cells which were able to expand and cause B cell aplasia. Vector copy number analysis of the liver demonstrated minimal off-target transduction in mice treated with the retargeted vector (unlike the high vector copy number seen with VSV-G treated mice).

In vivo generation of CD19 CAR-T cells by the systemic administration of a retargeted 4th generation lentiviral vector coupled with multiple innovative technologies has the potential to

dramatically widen the availability and accessibility of CAR-T treatment for both oncology and autoimmune indications.

P454

A novel engineered Treg platform that addresses, Treg stability, IL2 signaling and targeting, for the treatment of autoimmune diseases

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Clinical studies aiming to restore the immune homeostasis via in vitro sorted cultured Treg cell therapy were shown to be safe, however, several hurdles such as lack of stability, specificity and IL-2 signaling support, have hindered translation of Tregs into an effective treatment for autoimmune and autoinflammatory diseases. Treg identity can be impacted by the inflammatory environment which can skew the immunosuppressive phenotype towards proinflammatory effector function. Therefore, a safe and efficacious Treg product must thus be endowed with a locked Treg phenotype resistant to destabilizing cytokine cues. To enhance retention at the site of inflammation, a Treg cell therapy product can be armed with a TCR or CAR specific for a disease relevant antigen. Lastly, defects in IL-2 signaling associated with autoimmune conditions impair Treg homeostasis, indicating that providing IL-2 support will be critical for Treg stability, engraftment and function in patients.

Here, we report an Engineered Treg (EngTreg) cell therapy platform designed to address major limitations of sorted Tregs. EngTregs are produced from human autologous bulk CD4 T cells by RNA-guided nuclease (RGN)-mediated genome engineering to stabilize FOXP3 expression and thus Treg identity; allow for the replacement of endogenous TCR with an antigen-specific TCR; and introduce rapamycin-activated chemically induced IL-2 signaling complex (CISC).

Using bulk and single cell comparative transcriptomic analysis we find that EngTregs express higher levels of stability and ‘Core Treg’ genes. while expressing low levels of cytotoxic and proinflammatory genes (including GZMA and PERFORIN1) at steady state. Foremost, EngTregs when exposed to inflammatory cues maintain FOXP3 expression and acquire ability to produce IL-10. Utilizing type 1 diabetes (T1D) as an example, EngTreg expressing pancreatic islet antigen-specific TCR showed potent direct and bystander suppression of T1D patient T effector cells only in the presence of the cognate antigen. To enable EngTreg-selective IL-2 support, the cells are engineered to express a rationally designed receptor CISC, which promotes a full IL-2 signaling cascade upon activation by rapamycin. In vitro, CISC stimulation alone supports survival of EngTregs in the absence of IL-2, and further promotes proliferation with concurrent TCR signaling at half-maximal effective concentrations of rapamycin in the low nanomolar range. In vivo, CISC stimulation promotes engraftment of EngTregs in an NSG mouse host in a rapamycin exposure-dependent manner. Pharmacokinetic modeling of CISC activity vs. rapamycin exposures supports a rationale for subtherapeutic rapamycin dosing in patients to promote EngTreg engraftment and expansion in the IL-2 defective autoimmune environment. In summary, EngTregs are a novel cell therapy platform with a potential to restore tolerance in patients with autoimmune diseases.

P455

iTRUCK19.18: novel doxycycline-inducible IL-18 engineered CAR-T cells for the treatment of aggressive lymphomas

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Despite CAR-T cell therapy has shown long-lasting clinical responses in specific subsets of B cell acute lymphoblastic leukaemia or advanced lymphomas, there are several challenges that limit its effectiveness against solid tumors and high-risk hematological malignancies. These challenges include severe and life-threatening toxicities, antigen escape, restricted cell trafficking and CAR-T cells exhaustion, leading to frequent relapses following CAR-T infusion. Additionally, interactions between the host and the tumor microenvironment impair CAR-T cells function. Overcoming these obstacles requires strategies to enhance therapy efficacy and control associated toxicities. One proposed alternative is reinforcing conventional CAR-T cells to release cytokines (T cells redirected for antigen-unrestricted cytokine-initiated killing, TRUCKs). Although preclinical and clinical studies have shown promising results in patients with relapsed or refractory hematological neoplasms, continuous release of proinflammatory cytokines such as IL-12 or IL-18 can disrupt immune balance and lead to severe toxicities. To enhance the anti-tumor potency of CAR-T cells without compromising safety, we used the Lent-On-Plus system, a doxycycline-inducible transactivator-free expression system, to control IL-18 expression in αCD19 CAR-T cells (referred to as iTRUCK19.18). Through this approach, we generated iTRUCK19.18 cells and observed that IL-18 induction allowed for controlled release of proinflammatory cytokines while modulating T cells activation levels. Additionally, iTRUCK19.18 cells effectively polarized protumoral human primary macrophages (M2) towards an antitumoral phenotype (M1) in a doxycycline-dependent manner. This translated into increased anti-tumor activity of CAR-T cells, both *in vitro* and *in vivo*, against an aggressive Burkitt lymphoma model. In a more clinically relevant setting, we generated iTRUCK19.18 cells from patient T cells and observed that IL-18 release enhanced the elimination of primary B tumors. Interestingly, doxycycline administration reduced TOX expression in iTRUCK19.18 patient cells after serial tumor challenges, suggesting that IL-18 not only enhances killing activity but also protects CAR-T cells from differentiating into terminally exhausted phenotypes. To our knowledge, iTRUCK19.18 cells are the first CAR-T cells able to control IL-18 expression exogenously and may represent an alternative to the application of conventional CAR-T cells in patients with certain aggressive malignancies.

P456

Developing chimeric antigen receptor (CAR)-based immunotherapies for liver cancers

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Hepatocellular carcinoma (HCC) and hepatoblastoma (HB) are the two main types of liver cancers in adults and children, respectively. While the development of HB is largely due to germ-line mutations in specific cancer risk genes, HCC usually occurs due to chronic damage to the liver. Typically, early-stage liver cancers isolated to the liver can be cured by liver transplantation, advanced liver cancers however often have a poor prognosis due to primary or acquired multipharmacological resistance. Therefore, new treatment options are needed for these patients. Cellular therapy with chimeric antigen receptors (CARs) expressed on autologous T-cells as immune effector cells is a groundbreaking novel immunotherapy that is highly successful in B-cell malignancies targeting either CD19 or BCMA as antigens. To develop CAR T-cell therapy for liver cancer, we first screened two HB (HepG2, HuH6) and three HCC (HuH7, HLE, HLF) cell lines for expression of suitable target antigens that can be recognized by specific CAR constructs. Using flow cytometry, we demonstrated that none of the cell lines expressed relevant levels of CD44v6, while B7-H3, EpCAM or Glypican-3 (GPC3) were present at high antigen densities on HepG2, HuH6 and HuH7 cells, but absent on HLE and HLF cells. Epidermal growth factor receptor (EGFR) and HER2/ErbB2, two classical target antigens for solid cancer immunotherapies, were present at relevant densities on HLE and HLF cells and also at least two of the other lines. Next, we generated two to five 2nd generation CAR constructs for each target antigen in our standard 2ndgeneration lentiviral vector, which includes a CD34-derived hinge, CD28 transmembrane and costimulatory domains and a ζ-chain. To determine the cytotoxicities of the CAR T-cells, peripheral blood-derived human mononuclear cells were activated for two days with CD3/CD28, transduced with VSV-G-pseudotyped lentiviral vectors, purified to >90% transduced cells on MACS columns after staining with CD34 microbeads and then incubated with the cell lines at different effector to target cell ratios (3:1, 1:1; 0.3:1, 0.1:1, 0.03:1; 0.01:1). The CAR-mediated cytotoxicities were assessed after 16-20 hours, using either MTS- or luciferase-based 96-well read-outs. The results revealed that the killing efficacy of the CAR constructs was directly linked to the antigen expression levels and the affinity/avidity of the CARs. Based on the heterogeneity of target antigen expression usually present in solid cancers and the fact that the first clinical trials for CAR T-cell therapies in malignant brain tumor patients failed due to the outgrow of malignant cells that have lost or down-regulated the CAR targets, the next generation of CAR T-cells for solid cancers must be capable of targeting at least two antigens simultaneously. To this end, we are developing combinations of the scFvs for B7-H3 and GPC3 in either CAR and/or bispecific T-cell engager (BITE) formats for double antigen targeting of the malignant cells. These bi-specific targeting strategies must then be combined with efforts to armor the CAR T-cells against the hostile tumor microenviroenment.

P457

Autologous HSC-derived CAR-Treg gene therapy: a novel approach for the treatment of chronic autoimmune disease

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Transfer of regulatory T cells (Tregs) expressing chimeric antigen receptors (CARs) is a novel approach to the treatment of chronic autoimmune conditions. By introducing CARs that recognise autoantigens into Tregs, their immunosuppressive functions can be directed to allow immune homeostasis to be restored. While this approach has shown promise, it is undermined by questions regarding the stability of CAR-Tregs post transfer and their long-term efficacy. To address these concerns and to provide durable immunosuppressive function, we are developing a novel approach to the generation of CAR-Tregs using our autologous HSC gene therapy platform.

Using lentiviral vectors designed to transduce hematopoietic stem cells (HSCs), we have shown that CAR-Tregs can develop naturally *in vivo*, following transplantation of HSCs into conditioned recipients. This is achieved using engineered promoter elements that control and restrict CAR expression to the Treg compartment during natural T cell development. We demonstrate that such HSC-derived CAR-Tregs are stable, long lived and retain a Treg phenotype. Furthermore, *ex vivo* CAR stimulation by the specific ligand leads to Treg activation as shown by increased expression of CD25 and IL-10 secretion.

To achieve these specific outcomes, we first screened candidate promoter elements *in vitro* in both cell lines and primary murine/human T cells to evaluate promoter specificity and activity. In addition, the need for tertiary regulatory elements was found to be necessary to maximise promoter performance. A panel of regulatory elements was added to different promoter constructs and screened, *in vitro*, to identify optimal restriction of CAR expression. Following identification of optimal promoter and regulatory elements, HSCs were transduced with lentiviral vectors and transplanted into conditioned animals. To understand the level of CAR expression required to enhance Treg function, HSCs were transduced with increasing amounts of lentiviral vectors in order to correlate vector copy number (VCN) achieved in HSCs with CAR expression. The effect of increasing CAR expression on Treg function, phenotype and turnover were all assessed *ex vivo* following successful engraftment of transduced HSCs. Finally, to assess LV expression construct performance *in vivo*, we developed an inflammatory challenge model to robustly activate Tregs in specific lymphoid organs and non-lymphoid tissues, permitting analysis of antigen-specific CAR-Treg proliferation and recruitment to inflammatory tissues in mice.

This work represents the first proof of concept study for the development of CAR-Tregs *in vivo* following transduction of HSCs. For severe autoimmune diseases, our approach can overcome the limitations associated with adoptive CAR-Treg cell therapies. The realisation of the potential of HSC-derived CAR-Treg for immunomodulatory therapies represents an opportunity to provide lifelong treatments for a broad spectrum of severe chronic autoimmune disorders.

P459

Generation of Clonal Neoantigen Reactive T Cells (cNeT) Bearing Favourable Phenotypic Properties and Polyfunctionality Critical for Precision Adoptive Cell Therapies

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Tumour-infiltrating lymphocyte (TIL) therapies have demonstrated efficacy against multiple solid tumour types, including melanoma refractory to immune checkpoint blockade.

Several new studies support the notion that tumour neoantigens are key targets of tumour-infiltrating lymphocytes, and amongst those, clonal neoantigens (expressed by all tumour cells) best correlate with response to immunotherapy.

At Achilles Therapeutics, we have developed a bioinformatics engine (PELEUS™) able to identify clonal neoantigens and which combined with our VELOS™ manufacturing process have allowed us to generate clonal neoantigen reactive T cell (cNeT) products in multiple indications including metastatic melanoma and non-small cell lung carcinoma. Critically, the nature of the process used by VELOS™ allows in-depth quantification and characterisation of the active component of this next generation T cell therapy.

Reactive cNeT dose is used for product release; in addition, we identify the number of individual clonal neoantigen reactivities in each product (mean of 5.8 reactivities across 36 products). Comprehensive phenotypic analysis of cNeT products is routinely performed unveiling polyfunctional responses to antigen stimulation: cytokine release and activation marker upregulation, low expression of the inhibitory receptor PD-1, and high expression of functional receptors CD25 (IL-2R) and the tumour-tracking chemokine receptor CXCR3. This supports the functional and migratory potential of our cNeT cells. Lastly, single cell and bulk T cell receptor sequencing also enable the identification and characterisation of reactive T cells in the product and their tracking in the patient post-dosing.

Our unique ability to gather information about the reactivity, phenotype and functional responses in our cNeT products allows the development of clinically relevant potency assays for product release, is essential for rapid comparability of next generation products, and is critical for the understanding of the mechanisms underpinning resistance and response to cNeT therapy.

P460

Multiplex cell engineering of allogeneic anti-CD19 CAR T-cells with tuned silencing of HLA class I limits rejection by both CD8 T-cells and NK cells

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Autologous chimeric antigen receptor (CAR) T-cell therapy is an effective form of treatment for hematological malignancy. This personalized approach is however challenged by high manufacturing costs and complicated logistics, which limit widespread adoption. Moreover, durable treatment response and a lack of efficacy against solid tumors depends largely on the persistence of CAR T-cells, which not only relies on cellular potency, but also the ability to avoid rejection by the patient's immune system. To overcome this, the need to multiplex engineer a next generation CAR T-cells is essential, such that they can be provided as an off-the-shelf solution, manufactured at scale and designed with molecular features to enhance cellular persistence. We have developed a bimodal gene construct which co-expresses a miRNA-mediated gene silencing cassette in tandem with a CAR (miCAR). This approach not only facilitates highly efficient multiplex gene silencing, but also allows for "tunable" silencing of multiple target genes. Our aim was to develop allogeneic CAR T-cells with enhanced cellular persistence by tuning down the expression of HLA class I to limit rejection by both CD8 T-cells and NK cells. Gene constructs were first created to silence HLA-I to varying levels, of which three were cloned into a previously optimized miCAR construct expressing an anti-CD19 CAR (CAR19) and miRNA that silences TCR expression with high efficiency. Primary T-cells were modified via lentiviral vector transduction, expanded in G-Rex cell culture plates, and purified by depletion of TCR-expressing cells. *In vitro* characterization included flow cytometric immunophenotyping, cytotoxicity of CD19-expressing tumour cells, assays to demonstrate loss of alloreactivity, and hypoimmunogenicity testing with unmatched CD8 T-cells and NK cells in mixed lymphocyte reactions (MLRs). We successfully generated miCAR19 T-cells with complete TCR silencing and with tuned silencing of HLA-I over a range of 80-95%. While CAR functionality was maintained in short-term and recursive cytotoxic assays against tumor cells, hypoimmunogenic miCAR19 T-cells also resisted cytotoxicity by both CD8 T-cells and NK cells in MLR assays. Remarkably, there was a direct correlation between the level of HLA-I silencing and increasing cytotoxicity by NK cells. To conclude, we demonstrate use of the miCAR technology platform to multiplex engineer a novel class of hypoimmunogenic and allogenic CAR19 T-cells, offering a balanced protection from both CD8 T-cell and NK cell mediated rejection.

P461

Targeting WT1 in pediatric AML using TCRm CAR effector cells

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Acute myeloid leukemia (AML) is a rare and heterogenous malignancy in children and adolescents. Although ~80% 5-year survival rates are achieved with current treatment protocols for *de-novo* AML, the outcome of patients with relapsed or refractory disease in ≤40% of patients often remains poor. Cellular therapies with chimeric antigen receptor (CAR) immune effector cells are highly interesting alternative treatment strategies, however the most commonly expressed target antigens on AML blasts (CD33, CD123, CLL1) are also present at higher levels on healthy hematopoietic (progenitor) cells, thus restricting these CAR approaches to bridge-to-stem cell transplant settings. In the search for suitable targets for AML immunotherapy, the Wilms Tumor 1 (WT1) transcription factor, which is overexpressed in almost all types of leukemia and many solid tumors, appears to be an excellent target, as it is presented in peptides on the surface of specific HLA antigens. We therefore designed and cloned seven T-cell receptor mimic (TCRm) single chains, recognizing the immunogenic nine-mer $WT1_{126\text{-}134}$(RMFPNAPYL) on HLAA*0201, into classical second-generation CAR constructs. To test the antileukemic effect of these TCRm CARs, human effector lymphocytes (T- and NK-cells) were isolated from healthy donors and expanded and activated *in-vitro* by CD3/CD28 antibodies and IL-2 (T-cells) or IL-2 and IL-15 for the NK-cells. The cells were then transduced with lentiviral TCRm CAR constructs expressing CD28 co-stimulatory and transmembrane domains, CD3zeta chain and a truncated CD34-hinge which was utilized for both, detection of TCRm CAR expression and enrichment of the transduced effector cells *via* CD34 microbead-based magnetic separation (MACS). Enriched effector cells were then co-cultured with different AML lines and the lysis of target cells was assessed after 16 hours by live/dead staining using flow cytometry. Our results revealed that only four TCRm CAR effector cells efficiently killed $WT1^+$/$HLAA*0201^+$ leukemic blasts, two even with similar potencies as CARs against highly expressed surface antigens, e.g. CD33. In addition, the absent killing of the WT1 TCRm CAR effector cells against $WT1^+$/$HLAA*0201^-$ or $WT1^-$/$HLAA*0201^+$ cells demonstrated that the anti-leukemic potency of these CAR effector cells is both WT1 and HLA*A2 specific. *Proof-of-principle* studies revealed that a 48h pre-treatment of low HLA*A2 expressing cell lines with 500 U/ml IFN-γ1b increased the surface expression of MHC class I and specifically HLA*A2, which then translated into an increased killing efficacy by the WT1 TCRm CAR effector cells. Next, we lentivirally expressed HLA*A2 cDNA in cell lines genotypically negative for HLAA*0201 and performed cytotoxicity assays. While TCRm CAR effector cells were not able to kill $HLAA*0201^-$ blasts, cells overexpressing HLA*A2 were susceptible to WT1 TCRm CAR-mediated killing. Conclusively, our results revealed potent anti-leukemic effects for TCRm CAR T- and NK-cells against $WT1^+$/$HLAA*0201^+$ leukemic blasts, specific for both WT1 and HLA*A2 and sensitive to HLA*A2 expression levels. In future experiments, *in-vivo* cytotoxicity of these WT1 TCRm CAR effector cells will be tested in pediatric AML xenotransplantation models established in immunodeficient NSG mice. In addition, to assess on-target/off-tumor cytotoxicity of these living drugs, similar PDX models will be established in HLA*A2 knock-in NSG mice.

P462

ROR-1 specific CAR-T cells with CRISPR/Cas9-mediated glucocorticoid receptor knockout exert potent antitumor efficacy in advanced adrenocortical carcinoma

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Adrenocortical carcinoma (ACC) is a rare and aggressive endocrine malignancy with limited treatment options. The only curative option is complete surgical resection in an early tumor stage. Nevertheless there are high rates of local recurrences and distant metastases in ACC. In addition, ACC is characterized by endogenous glucocorticoid (GC) excess occurring in 60% of cases which is hypothesized to be one reason, why first clinical trials evaluating the potency of immune checkpoint blockade showed only modest results. Furthermore, the development of cell and immunotherapeutic treatment approaches has been impeded by the lack of cancer-associated antigens in ACC. Here, we report the identification of ROR-1 as a candidate target for the treatment of ACC and the preclinical assessment of a next-generation chimeric antigen receptor (CAR)-T cell product.

ROR-1 expression was evaluated at the mRNA and protein level in human ACC cell lines (n=5) and a representative cohort of human ACC specimen (n=197) by using qRT-PCR, qFACS, dSTORM, RNAscope single cell analysis and chromogenic immunohistochemistry (IHC). Our data show ROR-1 transcripts to be detected over background in 92.7 % of ACC samples at mRNA level (n=62) and in 91.1 % at protein level (n=135). ROR-1 mRNA expression was 2-fold higher in ACC as compared to normal adrenal glands (p=0.015) and upregulated 3-fold in metastases as compared to primary tumors (p=0.002). Furthermore, a strong correlation between ROR-1 expression and ENSAT tumor stage was found (p=0.009). ROR-1 was also highly expressed in all 5 ACC cell lines.

ROR-1 specific CAR-T cells (ROR-1-CART) were generated and their antitumor efficacy was tested using preclinical models of ACC. Our data show potent antitumor efficacy upon co-cultivation with ACC cell lines, including antigen-specific tumor cell lysis, cytokine secretion and T cell proliferation. Considering that ACC is well-known for its GC enriched inhibitory TME, we recapitulated these circumstances *in vitro* using steroidogenic ACC cell lines, exogenous supplementation of dexamethasone and 3D cell culture and observed a considerable decrease in antitumor efficacy elicited by ROR-1-CART. Therefore, we initially explored whether a combination treatment with steroid inhibitors or hGR modulators like metyrapone, ketoconazole, mitotane and relacorilant could be employed to restore antitumor efficacy of ROR-1-CART. However, we observed a significant mitigation of potency and antitumor killing, which could be attributed to a corticosteroid inhibitor-related downregulation of ROR-1 on ACC tumor cells. As a consequence, we sought to investigate the potential of desensitizing ROR-1-CART by CRISPR/Cas9-mediated genome editing of the hGR locus and found hGR^{KO}ROR-1-CART to exert identical antitumor efficacy under normal and immunosuppressive conditions (ROR-1-CART: 41.8% *vs* hGR^{KO}ROR-1-CART: 74.9 % specific lysis of NCI-H295R cells, E-T 1:1). Lastly, we

compared ROR-1-CART efficacy alone with hGR^{KO} and pharmaceutical blockade of GC effector functions and observed hGR^{KO}ROR-1-CART to be superior to a combined treatment approach.

Collectively these data show that ROR-1 is commonly and homogenously overexpressed in human ACC specimen. Preliminary results reveal enhanced efficacy of hGR^{KO}ROR-1 CART in 3D and *in vivo* models of ACC. Full data will be presented at the meeting.

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Pathogen-specific T cell receptor immunogenetic profiling in the context of adoptive immunotherapy

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The adoptive transfer of pathogen-specific T cells (pSTs) recognizing pathogen-derived peptides through their endogenous T-cell receptor (TR) represents an attractive treatment of viral and fungal infections post allogeneic hematopoietic cell transplantation. However, the TR repertoire of endogenous or/and *ex vivo* generated pSTs for immunotherapy has not yet been fully elucidated. We analysed the TR beta-chain gene repertoire by next-generation sequencing (NGS) of two immunotherapy products under evaluation in phase I/II clinical trials: i) tri-virus specific T cells (*tri-VSTs*) targeting cytomegalovirus (CMV), Epstein-Barr virus (EBV) and BK virus (BKV) (EudraCT:2014-004817-98) and ii) pentavalent-specific T cells (*penta-STs*) additionally targeting adenovirus (ADV) and the fungus *Aspergillus fumigatus* (AF) (EudraCT:2020-004725-23). GMP-grade tri-VSTs and penta-STs were manufactured after exposure of peripheral blood mononuclear cells (PBMCs) from immunocompetent donors to CMV, EBV, BKV, ± AdV, ± AF overlapping peptides and 10-day culture. The specificity of donor-derived cell products and patient-derived PBMCs was assessed by IFN-γ Elispot. TCRB sequencing was performed in the whole T cell products (tri-VSTs n=2, penta-STs n=4), their corresponding pathogen-specific cell subsets (n=26) post immunomagnetic IFN-γ enrichment, and in patient PBMCs (n=26). Immunogenetic analysis was performed by RT-PCR amplification of TRBV-TRBD-TRBJ rearrangements according to the BIOMED-2 protocol and paired-end NGS (Miseq/NextSeq). After length and quality filtering, the NGS sequences were submitted to IMGT/HighVQUEST for annotation. Metadata analysis and clonotype computation (TRB rearrangements using the same TRBV gene and identical CDR3 amino acid sequence) were based on a validated purpose-built bioinformatics platform (*tripr*). Tri-VSTs and penta-STs provided a diverse TR repertoire consisting of 6,580-33,863 unique clonotypes/sample (median:22,530) and in terms of clonality the median frequency of the major clonotype was 6.48% (range:2.23%-20.7%). The clonotypes identified in the enriched subpopulations were subjected to strict filtering: i) ≥10 read counts, ii) higher frequency in the enriched subpopulations over the unselected product as defined by the greater frequency (fold increase) of each post-enrichment clonotype than the median fold increase of all enriched clonotypes. The applied criteria resulted in 9,799 clonotypes, of which 8,749 were present in a single specific-cell fraction, arguably suggesting that they are pathogen-specific (CMV-specific:2,768, EBV-specific:1,763, BKV-specific:2,003, ADV-specific:1,363,

AF-specific:852). Indeed, several of those identified virus-specific clonotypes could be tracked *in vivo* in two patient-derived PBMCs up to 15 weeks post tri-VST infusion with ranging frequencies 0.002%-12.7% (Pt1: 32 CMV-specific, 25 EBV-specific and 29 BKV-specific & Pt2: 20 CMV-specific, 31 EBV-specific and 31 BKV-specific clonotypes). Importantly, their expansion *in vivo* was inversely correlated with the corresponding viral load and positively correlated with the frequency of circulating virus-specific T cells. Overall, our findings elucidate the diverse immunogenetic profile of *ex vivo* generated pSTs, identify potential pathogen-specific clonotypes conferring protection against infections and provide a novel method to track *in vivo* the infused pSTs in patients receiving adoptive immunotherapy. Prospectively, identifying optimal TRs that mediate clinical responses may serve as a stratification tool for patients at risk for suboptimal responses and help select the best candidates for adoptive immunotherapy.

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Riboswitch-regulated chimeric antigen receptor (RiboCAR) enhances CAR-T cell anti-cancer efficacy

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Chimeric antigen receptor (CAR)-T cell therapy is a promising therapy against cancer. However, the uncontrolled CAR expression causes severe CAR-T cell-associated toxicity and CAR-T cell exhaustion, limiting the success of this living drug. Here, we present the development of RiboCAR, a mammalian synthetic riboswitch-regulated CAR expression via small molecule inducer. Unlike previously reported regulatable CAR platforms that utilize viral protease or chemical-induced protein dimerization, RiboCAR contains an RNA ON riboswitch in the coding sequence of a CAR transgene, in which the aptamer functions as a sensor for a specific novel small molecule inducer. The expression level of the CAR gene with the riboswitch completely depends on the presence of the riboswitch inducer, with undetectable CAR in the absence of the small molecule and significant CAR expression that is higher than constitutively active CAR upon maximal small molecule induction. The induced CAR expression diminished after withdrawal of the small molecule inducer. Further, CAR expression is titratable in response to the levels of the small molecule inducer. Consistent with small molecule induced expression of the CAR molecule, CAR triggered-activation of CAR-T cells is also controlled by the small molecule inducer. More importantly, T cells with RiboCAR showed delayed exhaustion during expansion in the absence of small molecule inducer and enhanced target cell-stimulated T cell activation and anti-cancer cytotoxicity in the presence of small molecule inducer, when compared with T cells constitutively expressing CAR. With a bioavailable small molecule inducer, the RiboCAR-T cell activity can be precisely tuned and "remotely" controlled *in vivo*, thus improving the efficacy and safety of CAR-T cell therapy.

P465

Targeting PRAME using TCRmimic CAR Effector Cells: A Promising Approach for the Treatment of Pediatric AML

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The treatment of relapsed or refractory (R/R) pediatric acute myeloid leukemia (AML) patients is highly challenging. These AML patients are heavily pre-treated and often do not achieve the minimal residual disease levels, necessary to be eligible for allogeneic hematopoietic stem cell transplantation (HSCT) as the only still curative treatment option. Here, alternative curative or *bridge-to-transplant* strategies are needed. Cellular-based immunotherapies have been recently proven highly successful as valid treatment options in B-cell-lineage malignancies using CD19- or BCMA-directed autologous chimeric antigen receptor (CAR) T-cells. However, due to the huge intra-patient and inter-patient heterogeneity of AML, finding a universal cancer-specific target in AML has proven to be challenging. Preferentially expressed antigen in melanoma (PRAME) is a cancer-testis antigen that is highly expressed in many solid tumors and hematological malignancies, including AML, but is completely absent in hematological cells and rarely expressed at low levels in extramedullary normal tissues. Using T-cell receptor mimic (TCRm) antibodies, it is possible to target immunogenic PRAME peptides presented in the context of human leukocyte antigen (HLA) molecules on the surface of cells. To this end, we cloned two TCRm single chains against HLA-A*02 restricted epitopes PRA300-309 (ALYVDSLFFL) and PRA435-443 (NLTHVLYPV) into a second-generation CAR construct, containing CD28 transmembrane and co-stimulatory domains, the CD3ζ chain, and a CD34-derived hinge domain. We then transduced primary T- or NK-cells with these CARS, using lentiviral expression systems, and utilized CD34 magnetic beads to enrich the transduced effector cells on a magnetic cell separator. To test the cytotoxicity of these highly purified CAR immune effector cells, we co-cultured the cells with AML blasts for 16 hours and then assessed the percentage of the lysis of the target cells by live/dead staining and flow cytometry. TCRm CAR NK-cells killed PRAME$^+$/HLA-A*02$^+$ target cells OCI-AML-2 and THP-1 as effective as the classical CAR NK-cells directed against extracellular epitopes of CD33, CD123, or CLL1. In addition, TCRm CAR NK-cells did not eliminate the PRAME$^-$/HLA-A*02$^-$ U-937 cells; however, they showed very efficient killing after we stably introduced HLA-A*2 and PRAME cDNAs into these cells. These results proved the specificity of TCRm CAR construct for PRAME$^+$/HLA-A*02$^+$ cells. Since we detected lower cytotoxicity levels in TCRm CAR T-cells compared to the NK-cells, we planned to enhance the cytotoxicity of the T-cells by up-regulating the expression of the endogenous HLA-A*2 protein. To this end, we pre-treated the target cells with 500 U/ml IFN-γ1 for 48h hours and showed that surface HLA-A*02 expression significantly increased. This treatment also led to increased killing of the leukemic cells by TCRm CAR T-cells. In conclusion, our TCRm CAR T- and NK-cells showed a high anti-leukemic potency against PRAME$^+$/HLA-A*02$^+$ target cells, specific for both PRAME$^+$ and HLA-A*02$^+$ and sensitive to HLA-A*02 expression levels. Future experiments will evaluate the effect of treatment with clinically used demethylation agents on the PRAME expression levels and TCRm CAR T- and NK-cell cytotoxicity. In addition, *the in vivo* cytotoxicity of these TCRm CAR effector cells will be tested in pediatric AML xenotransplantation models established in immunodeficient NSG mice.

P466

CD44 molecule expression negatively regulates the in vitro and in vivo antitumor efficacy of HER2-specific CAR-NK cells

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HER2-targeted monoclonal antibodies improve the outcome for advanced breast cancer patients; however, a great percentage of patients experience tumor relapse despite persistent antigen expression. Overexpression of CD44/hyaluran and MUC4 matrix components results in reduced trastuzumab binding and is clinically associated with poor prognosis. Immune cell therapy with adoptively transferred HER2 specific CAR T cells is an engaging option to improve outcomes for patients with advanced trastuzumab-resistant HER2-positive cancer. In our preclinical trial, HER2-specific CAR T cells recognized and efficiently killed trastuzumab resistant JIMT-1 and MDA-HER2 xenograft tumors. However, serious side effects and impracticable production have emerged as potential limitations in human clinical trials. We believe that natural killer cells can equivalently substitute T cells as effectors and at the same time could overcome these limitations. Thus, in this project, we propose to generate human NK cells that HER2 tumor antigen and test their in vitro and in vivo effector functions in CD44 negative (mAb therapy sensitive) and positive (mAB therapy resistant) HER2 expressing tumors. HER2-CAR NK-92 cell lines were generated by retroviral transduction on Retronectin coated plate. Viral particles encoded αHER2_scFv-CD3z (I. generation), αHER2_scFv-CD28-CD3z or αHER2_scFv-41BB-CD3z (II. generation) and αHER2_scFv-CD28-41BB-CD3z (III. generation) CAR constructs. Following FACS sorting all products have shown > 90% HER2-CAR positivity. First, we performed conventional immunological assays to investigate in vitro activation and cytolytic efficacy of HER2-CAR NK-92 cells in the presence of HER2+/CD44- N87 gastric cancer and HER2+/CD44- JIMT-1 breast cancer cell lines. To explore whether CAR NK-92 cells can access ECM-masked HER2+ cells we compared their in vivo effector functions in subcutaneous N87.ffLUC and JIMT-1.ffLUC xenograft models in which effector cells were administered i.v. on day 14 after tumor cell inoculation and then biweekly. We found that all generations of HER2-specific CAR-NK cells successfully recognized and killed HER2 positive target cells. In vitro in 2D cell culture, CD44 expression did not affect killing efficiency. HER2-CAR constructs containing the 41BB costimulatory endodomain showed better cytolytic efficiency than their counterparts without 41BB. In vivo, all generations of HER2-specific CAR NK-92 cells recognized and induced antitumor effect against CD44-negative N87 xenografts. Third generation CAR-NK-92 cells performed the best in this model. However, none of the HER2-CAR NK-92 cell lines could induce a prolonged anti-tumor effect in CD44-positive JIMT-1 xenografts. Our results suggest that the expression of CD44 not only impairs the efficacy of NK cell mediated antibody therapies (ADCC effect) by epitope masking but also directly inhibits the penetration of target-specific CAR-NK-92 cells. In our prior findings, this inhibitory effect was not observed on CAR T cells. These results highlight that T cells cannot be excluded from the therapeutic protocols in mAb-resistant solid tumor trials. In our future research, we would focus on systems that can involve NK and T cells together in the antitumor response.

P467

Developing universal *off-the-shelf* CD123 and CLL1 dual targeting tandem CAR NK-cells for the treatment of AML

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Pediatric acute myeloid Leukemia (AML) is a rare and heterogeneous disease, for which continuous improvement of risk-adapted treatment approaches over the last 30 years has led to 5-year survival rates of ~80%. Despite tremendous efforts, first-line treatment strategies are often not successful, as up to 40% of patients treated today face relapsed or refractory (R/R) AML. In this situation, allogeneic hematopoietic stem cell transplantation (HSCT) is the only realistic chance for long-term cure and survival. However, for HSCT to be successful, it is imperative that the residual number of blasts in the bone marrow is as low as possible (<5%). Immunological therapies with chimeric antigen receptor (CAR) immune effector cells are highly interesting alternative treatment strategies, however, two frequently overexpressed target antigens on AML blasts (CD123, CLL1) are also present at higher levels on healthy hematopoietic (progenitor) cells, thus restricting these cellular immunotherapies to *bridge-to-stem cell transplant* settings. Therefore, the aim of this project was to develop a universal *off-the-shelf* adoptive cellular product targeting CD123 and CLL1 with improved safety features and limited survival *in vivo*, suitable for *bridge-to-transplant* settings to render AML R/R patients eligible to undergo HSCT. To this end, we systematically compared two different CD123 and five different CLL1 single chain variable fragment (scFv) clones as single targeting moieties in 2nd generation CAR constructs on human T- and NK-cells for their ability to efficiently eradicate CD123+ and/or CLL1+ AML blasts *in vitro*. All CAR constructs are expressed in lentiviral vectors under the control of the MPSV promoter and contained a CD28-derived transmembrane and cytoplasmic region. We included a hinge region of 99 amino acid sequence derived from human CD34, that efficiently binds the CD34 antibody QBEND-10 and therefore allows to readily assess the expression levels of the CARs by flow cytometry and to select the transduced cells by MACS. From the best two CLL1 candidates, we then systematically designed CD123 and CLL1 tandem CAR combinations, using three different linkers. Importantly, the CD34 microbead-assisted enrichment of lentivirally transduced CAR T- and NK-cells was as efficient in tandem CARs as in single CAR constructs. With these dual targeting constructs, we generated pure and highly cytotoxic CD123/CLL1 tandem CAR NK-cells under GMP-compatible *in vitro* expansion conditions that show efficient and specific killing of CD123+ and/or CLL1+ AML cell lines as well as primary pediatric AML blasts *in vitro*. The best two tandem CAR constructs were selected to additionally express cassettes for either soluble or tethered IL-15 to extend the survival of CAR NK-cells. The final steps will be to test the ability of these four tandem CAR candidates *in vivo* in immunodeficient mice to control the growth of CD123+ and/or CLL1+ blasts of either established AML cell lines or primary xenografts of our pediatric AML patients. Therefore, this study provides the *in vitro* development of a universal *off-the-shelf* CD123/CLL1 tandem CAR NK-cell product that can be used in *bridge-to-transplant* settings to render the R/R AML patients eligible to undergo HSCT, the only potentially still curative therapy.

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Multiplex silencing of six molecules endows allogeneic anti-CD19 CAR T-cells with properties of enhanced cellular potency and limited rejection

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Chimeric antigen receptor (CAR) T-cell therapy is an accepted form of treatment for haematological malignancies. Despite impressive clinical achievements, several challenges remain, including a lack of efficacy against solid tumour cancers and limited accessibility to these treatments. To overcome this, a next generation of CAR T-cells is required, such that they can be provided as an off-the-shelf (allogeneic) solution, capable of durable treatment outcomes. The latter correlates directly with the persistence of the allogeneic CAR T-cells, which relies not only on the potency of these cells, but also on their ability to avoid rejection by the host immune system. We have developed a novel bimodal gene construct for simultaneous CAR expression and microRNA (miRNA) mediated gene silencing (miCAR). Using this construct, our aim was to multiplex engineer anti-CD19 CAR T-cells with silencing of six cell surface receptors, namely the T-cell receptor (TCR), human leukocyte antigen class I (HLA-I), CD52, PD1, TIM3 and TIGIT. Silencing of first three mentioned receptors facilitates the off-the-shelf provision of allogeneic CAR T-cells with the added benefit of being able to employ strategies to limit immune rejection. Functional silencing of the inhibitory receptors (PD1, TIM3 and TIGIT) aims to enhance cellular potency. Primary T-cells were modified via a single lentiviral vector transduction step to engineer miCAR T-cells, which were subsequently expanded in G-Rex cell culture plates, and in final step purified by depletion of TCR-expressing cells. Characterization of miCAR T-cells included flow cytometric immunophenotyping, functional activity against CD19-expressing tumor cells, and mixed lymphocyte reactions (MLRs) to assess for loss of alloreactivity of TCR-silenced cells and protection from allogeneic CD8 T-cell and NK cell mediated rejection. We demonstrate robust and reliable production of CAR T-cells with >99% TCR negativity and efficient silencing of all targeted receptors, while maintaining a favourable immunophenotypic profile with 50-70% being of a naïve phenotype (n=3 donors). Functionally, the multiplex engineered miCAR T-cells were shown to be more potent against CD19-expressing tumour cells when compared to control anti-CD19 CAR T-cells with TCR-only silencing. Finally, we show negligible activation of miCAR T-cells in CD3 activation assays, and protection against both CD8 T-cell and NK cell mediated depletion. The latter is supported by the notion that residual HLA–I expression (10-25%) is sufficient to protect CAR T-cells from the missing-self recognition response of NK cells. We therefore show efficient multiplex engineering of functionally active, non-alloreactive and hypoimmunogenic CAR T-cells with silencing of six target genes.

P469

Overnight-manufactured UltraCAR-T® cells with first-in-class miRNA-based PD1 blockade demonstrates enhanced polyfunctionality and sustained cytotoxicity against hematological and solid tumors

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CAR-T cell therapy has demonstrated durable efficacy in certain hematological malignancies but limited success in the suppressive environment of solid tumors. There is growing precedence for combination of CAR-T plus checkpoint inhibitors, such as anti-PD1 or PD-L1 antibodies, to reduce the suppressive signaling of the tumor microenvironment that leads to CAR-T exhaustion and poor efficacy. Moreover, traditional CAR-T manufacturing in centralized GMP facilities is expensive and labor-intensive, as a result of utilizing viral vectors and need for *ex vivo* cell activation and extensive expansion to obtain sufficient cell numbers for treatment. The lengthy production time can lead to treatment delays as well as progression of the CAR-T cells toward an exhausted phenotype. The addition of systemic checkpoint inhibition to traditional CAR-T therapy further complicates the treatment as well as increases toxicity risk and cost.

The UltraCAR-T platform overcomes the limitations of traditional CAR-T manufacturing with an advanced non-viral multigenic delivery system and an overnight, decentralized manufacturing process for administration of autologous CAR-T cells one day after gene transfer. Here, we describe PRGN-3007, based on next generation UltraCAR-T consisting of an intrinsic miRNA-based PD1 blockade module to counteract CAR-T cell exhaustion; a CAR targeting the receptor tyrosine kinase-like orphan receptor 1 (ROR1) which is overexpressed in various hematological and solid tumors; membrane-bound IL-15 for enhanced *in vivo* expansion and persistence; and a kill switch for improved safety profile. All components are encoded within a single non-viral transposon, thereby ensuring a homogenous cell product. The miRNA-based PD1 blockade avoids complex and expensive gene editing techniques and is localized to UltraCAR-T cells to prevent CAR-T exhaustion without the need for anti-PD1 combination.

Compared to control ROR1 CAR-T cells lacking PD1 blockade, PRGN-3007 demonstrated sustained and specific intrinsic downregulation of PD1 *in vitro* and *in vivo*, enhanced CAR- T cell cytokine polyfunctionality, cytotoxicity, and inflammatory cytokine release upon co-culture with ROR1$^+$/PD-L1$^+$ hematologic and solid cancer cell lines. Furthermore, PRGN-3007 maintained cytotoxic potential even after repeated challenges with ROR1$^+$/PD-L1$^+$ tumor cells in contrast to control ROR1 CAR-T cells. In an aggressive xenograft model of mantle cell lymphoma with high PD-L1 expression, a single administration of PRGN-3007, only one day after gene transfer, effectively reduced tumor burden and significantly improved overall survival compared to control ROR1 CAR-T cells. PRGN-3007 demonstrated rapid cell expansion, long-term persistence, and a predominantly central memory and stem cell-like memory phenotype. Importantly, inclusion of the miRNA-based PD1 blockade improved all measured functions of the UltraCAR-T cells without off-target gene silencing, activation of alternate checkpoint pathways, or increased toxicity, indicating miRNA as an attractive method for intrinsic PD1 blockade. PRGN-3007 next generation UltraCAR-T is currently under clinical investigation for advanced ROR1+ hematological and solid tumors (NCT05694364).

P470

Design of CAR T cells targeting metastatic colorectal cancer

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Chimeric antigen receptor (CAR) T cell therapy proved to be an efficient weapon for tumor therapy, especially in hematological malignancies like relapsed/refractory B-cell acute lymphocytic leukemia, non-Hodgkin lymphoma, and multiple myeloma. However, there are still many obstacles to be overcome for the use of CAR T cell therapy in solid tumors. One of the main obstacles is the lack of specific tumor target antigens. The focus of our project is the design of CAR T cells targeting metastatic colorectal cancer (mCRC). To reach this aim, we generated and screened new monoclonal antibodies (mAbs) specifically targeting metastatic colorectal cancer stem cells (mCRC-SCs), an important cell population in tumor progression. During the first phase of the study, we immunized BALB/c with primary mCRC-SCs. Then we generated a random hybridoma library by performing 36 fusions that were directly cloned, we selected the hybridomas secreting tumor-specific mAbs by high-throughput flow cytometric (FACS) screening and selected 203 hybridomas showing binding on mCRC-SCs, but not to PBLs. The mAbs with IgG isotype were purified using the ÄKTA pure chromatography system and immunohistochemistry (IHC) was performed to select mAbs that specifically bind CRC and not to normal mucosa or other healthy human tissues. Until now, two mAbs called 7D7.2 and 2A1.3 passed this stringent selection. These antibodies were further tested by flow cytometry using couples of freshly dissociated primary tumor tissue and freshly isolated normal mucosa from the same patient.
In the second phase of the project, we sequenced the variable regions of the antibodies and used the sequence information for the generation and functional testing of tumor-specific scFvs. These were then used to generate novel CAR constructs and to produce novel CAR T cells. More in detail we amplified the variable regions of the mAb using 5'-RACE PCR, cloned them inside the pCR-Blunt II-TOPO vector, and sequenced the inserts using Sanger sequencing. To enhance the chance to find the scFv-orientation with the best expression and binding characteristics, we designed 4 individual artificial genes containing two different scFv chain orientations (VH-Vk and Vk-VH), connected with two different linkers (short and long). The genes were cloned in the pcDNA3.1 (-) vector for transfection and protein expression in 293T cells. The resulting scFv-Fcs were purified and tested by flow cytometry. For 7D7.2, we found the VH-Vk orientation with long linker as being most efficient in binding the target cells. Thus, we used this orientation to produce chimeric antigen receptors differing in the length of the hinge region (CD8 hinge vs IgG CH2-CH3) and the intracellular signaling portion (4.1BB-zeta vs. CD28-4.1BB-zeta). For the gene transfer in primary human T cells, we used a lentiviral vector system based on pLENTI6 (Invitrogen). We isolated T-cells from different donors, activated and transduced them, and then performed in vitro T cell killing assays toward tumor target cells. Currently, we set up the identification of the antigens bound by the mAbs 7D7.2 and 2A1.3 using two-step coimmunoprecipitation (TIP) purification coupled with mass spectrometry.

P472

Development of physiological and inducible TRUCKs targeting pancreatic solid tumours

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In contrast to non-haematological cancers, solid tumours have remained resistant to Chimeric Antigen Receptor (CAR) T-cell therapy. On the one hand, commercial CAR-T cells are generated using retroviral vectors containing strong promoters, which lead to tonic signalling, overstimulation, and premature exhaustion, reducing the treatment efficacy and increasing toxicities. Several studies have demonstrated that when the CAR expression aligns with the natural behaviour of the T cell receptor (TCR), the fitness of CAR-T cells is significantly enhanced, which translates into a higher antitumoral efficacy. On the other hand, solid tumours present additional barriers, including the lack of tumour-specific targets, the difficulty in traffic and infiltration into the tumour, and the *milieu* of immunosuppressive factors that operate within the tumour microenvironment (TME). Here, we propose that the use of a TCR-like promoter-driven CAR-lentiviral vector (LV), together with LentOnPlus harboring IL-18, could avoid CAR-T cell exhaustion and re-shape the TME to allow CAR-T cell killing. We generated a heterogeneous CD19-expressing metastatic pancreatic ductal adenocarcinoma model, and our results showed that TCR-like promoter-driven anti-CD19-CAR-T cells maintained improved properties after the elimination of target cells *in vitro*. Additionally, when we generated inducible TRUCKs capable to release IL-18 in the presence of dox (αCD19-iTRUCK-IL-18), they exhibited enhanced antitumor potency in the presence of the inductor. Importantly, αCD19-iTRUCK-IL-18 cells demonstrated an increase in T_N, T_{SCM} and T_{CM} populations, and the release of IL-18 retained exhaustion upon serial tumour challenges. Finally, to assess efficacy of the inducible TRUCKs in solid tumours, we established an orthotopic CD19+ pancreatic tumour mouse model. In this model, the inoculation of αCD19-iTRUCK-IL-18 cells showed a robust antitumor response that was enhanced when combined with oral administration of doxycycline. These results show that the endogenous control of CAR expression through TCR-like promoters, together with the exogenous control of IL18 release, constitute a promising strategy to generate safe and efficient CAR-T cell products against solid tumours.

P473

CD8-targeted lentiviral vectors for in vivo generation of CAR T cells to treat peripheral T cell lymphoma

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Angioimmunoblastic T cell Lymphoma (AITL) is a rare cancer, which has no curative treatment. We recently generated a transgenic mouse model, which developed a $CD4^+$ follicular T cell-like lymphoma, mimicking AITL (*Mondragon* et al., *Cancer Cell, 2019*). In the case of AITL the malignant driver cell is a T cell and more specifically a $CD4^+$ T cell. Chimeric Antigen Receptor (CAR) T cells seems to emerge as a promising technique for cancer therapies especially for leukemia and lymphomas. Therefore, we developed as a new therapeutic option for AITL, a CD8-targeted lentiviral vector (LV) that allows anti-CD4 CAR expression only by $CD8^+$ T cells. It is of utmost importance to target exclusively the $CD8^+$ T cell population for expression of the CAR to avoid malignant T cell induction. A DARPin specific for mCD8alpha was displayed at the vector surface, allowing a specific recognition of $CD8^+$ T cells. Moreover, to avoid life-long immunosuppression, the CAR construct is co-expressing a safety switch, to permit CAR T cell elimination *in vivo*. We showed here that $CD8^+$ T cell transduction is specific and the expression of the CAR induced $CD4^+$ T cell death in murine WT splenocytes or murine T cell lymphomas *in vitro*. Currently, we are exploring the specificity and efficacy of CD8 targeted LVs encoding the anti-CD4 CAR *in vivo* in our T cell lymphoma preclinical mouse model.

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Development of all-in-one lentiviral vector for CAR-T therapy that enhances anti-tumor response and reduces risk of CRS and ICANS

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CAR-T cell therapies have shown effective antitumor response against hematological malignancies, and several CAR-T cell products targeting CD19 or BCMA have already been approved. However, since relapse occurs frequently and the therapeutic effect on solid tumors is limited, further development of CAR-T cells with long-lasting antitumor response is required. On the other hand, suppression of side effects must also be considered. Cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) are the most frequent side effects, and enhancing the antitumor effect could lead to an increase in these risks. Therefore, it is necessary to develop CAR-T cells with high antitumor response while suppressing serious side effects.

Recently, we have developed a novel cytokine receptor that captures IL-6 and IL-1, which cause CRS and ICANS, respectively. Expression of the novel cytokine receptors in CAR-T cells is

expected to reduce side effects due to absorption of cytokines secreted by macrophages. In our novel cytokine receptor, we used the extracellular domain of IL-6 receptor alpha chain fused to domains of GP130 to increase the affinity to IL-6, and linked the transmembrane and intracellular domain of the mutated IL-7 receptor alpha chain, which constitutively activates the JAK/STAT pathway while attenuating Akt signaling. Co-introduction of CAR and the chimeric cytokine receptor using individual viral vectors has resulted in long-term in vivo persistence of CAR-T cells and improved therapeutic efficacy. In addition, IL-1 Receptor Type 2 was co-expressed to absorb IL-1 beta in CAR-T cells, and the chimeric cytokine receptor-expressing CAR-T cells could efficiently reduce both IL-6 and IL-1.

In order to apply this technology to the clinic, it is necessary to develop an all-in-one vector that can highly express individual genes in terms of therapeutic efficacy and cost efficiency. In this study, we have developed all-in-one lentiviral vector to improve and optimize the expression level of each gene. First, we generated lentiviral vectors with various internal promoters and selected the MND promoter as a suitable promoter for CAR-T cells. We then enhanced expression level and function of receptors by codon optimization. When expressing multiple factors in a single vector, the expression balance of each factor is important. Vectors were constructed by switching the order of CAR, chimeric IL-6 receptor and IL-1 receptor to determine the most functional design. Furthermore, we determined whether insertion of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) into this vector would enhance its function.

Administration of T cells infected with the all-in-one lentiviral vector to a solid tumor-bearing mouse model resulted in significantly prolonged in vivo survival compared to conventional CAR-T cells, leading to almost complete tumor regression.

This technology is expected to be widely applicable as a versatile CAR-T therapeutic platform, regardless of the target antigen, and to enhance its efficacy and safety.

P475

Myelin Oligodendrocyte Glycoprotein (MOG)-CAR-Tregs – a novel approach to treat multiple sclerosis.

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An imbalance between Tregs (regulatory T cells) and inflammatory responses can result in excessive inflammation, breakdown of tolerance and autoimmune disease. Multiple sclerosis (MS) is an immune mediated disease affecting the central nervous system (CNS) with the immune system attacking myelin sheaths leading to neuronal death. Data suggests that the frequency of Treg and some Treg populations might be altered in MS patients, leading to aberrant immune response. Current treatments mostly manage inflammation and symptoms of MS. Disease-modifying drugs that can restore Treg homeostasis and immune tolerance are needed. Immunotherapies using adoptive transfer of Tregs is an exciting approach. The introduction of a chimeric antigen receptor (CAR) targeting the CNS is a strategy to increase Treg potency.

We developed a MOG-specific CAR-Treg. MOG is expressed on the outer membrane of myelin sheath, making it an ideal target for CAR-Treg therapy. Our lead candidate is a 2nd generation

CAR, composed of an anti-MOG scFv screened from a large human library. In vitro, we demonstrated CAR-dependent functionality by using suppression and activation assays using mouse spinal cord. Additionally, the MOG-CAR-Tregs have very low tonic signaling with a desirable signal-to-noise ratio. Our data also suggest that MOG-CAR-Tregs might be efficient at promoting differentiation of oligodendrocytes in vitro and remyelination in an ex vivo mouse brain slices model. Moreover, in vivo, MOG-CAR-Tregs were significantly efficacious in a passive MS mouse model.

In summary our data suggest that we identified a promising CAR-Treg product candidate to treat MS patients.

P476

Unleashing genetically engineered CAR-NK cells against pan-cancer antigen B7-H3 to treat gynaecological cancers

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Cervical and ovarian cancers are severe gynaecological malignancies that threaten women worldwide, with nearly 1 million diagnoses yearly. Despite significant efforts in preventing the development of these diseases, such as anti-HPV vaccine for cervical cancer and advanced preventive screening, both cancers still have concerning mortality rates. In light of the urgent need for new therapies, immunotherapy appears as a promising option and has been applied in gynaecological oncology through checkpoint inhibitors, adoptive cell therapy, and cancer vaccines.

To combat these cancers, we applied genetically engineered NK cells to create "off-the-shelf" cell-based immunotherapies. Therefore, NK-92 cells and primary NK cells were modified with self-inactivating (SIN) alpharetroviral vectors expressing chimeric antigen receptors (CAR) that target the immune checkpoint molecule B7-H3, which contributes to immune evasion and is preferentially expressed in cancer cells versus healthy tissues. In line with earlier reports, we observed elevated B7-H3 levels in ovarian and cervical cancer cell lines as well as in primary cultures derived from patient samples. However, the biggest challenge in immunotherapy is often the tumour microenvironment (TME), which includes various cellular and non-cellular components that interact with tumour cells, significantly impacting their progression and response to treatments. To overcome the TME and induce a pro-inflammatory environment, we additionally applied TRUCK ("T cells redirected for antigen-unrestricted cytokine-initiated killing") principles to further modify CAR-NK cells with an "all-in-one" alpharetroviral vector system designed to constitutively express the anti-B7-H3 CAR, and inducibly express various immunomodulators, such as the apoptosis inducer TRAIL. Thus, we generated CAR- and TRUCK-NK cells targeting B7-H3 and characterised the protein expression level of the anti-B7-H3 CAR and quantified vector integration sites.

Cytotoxic activity against cancer cell lines and primary cancer cells expressing B7-H3 was assessed in 2D co-culture experiments via flow cytometry and fluorescence-based cytotoxicity

assays using an automated imaging analyser. To better recapitulate the immediate environment of highly complex tumours and their stroma, we developed 3D tumour spheroid and organoid models for cervical and ovarian cancers and tested the anti-cancer activity of engineered NK cells on these 3D tumour models. Anti-B7-H3 CAR- and TRUCK- NK cells effectively destroyed tumour spheroids even at low effector target ratios, exhibiting cytotoxic activity similar to 2D cell culture monolayers. In contrast, unmodified NK cells showed significantly lower anti-tumour activity.

Our data demonstrate the high efficacy of modified CAR- and TRUCK-NK cells to target ovarian and cervical cancer cells and the TME. Moreover, we identified B7-H3 as a promising candidate for improved treatment of gynaecological cancers. In addition to our well-established vector systems tailored for efficient NK cell modification, we adopted a robust platform that employs automated cytotoxicity assays on 3D tumour models to evaluate cell therapies against novel targets, which can also be applied to test combinations of personalised therapy options.

P477

Leveraging an RNA-based Lipid Nanoparticle (LNP) Gene Writer System to generate functional Chimeric Antigen Receptor T cells (CAR-T) that clear tumors *In Vitro* and *In Vivo*

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Chimeric Antigen Receptor (CAR) T-cell therapies have shown the ability to eradicate very advanced leukemias and lymphomas in cancer patients. Despite CAR T-cell therapies becoming mainstream, significant challenges exist: (1) long-term survival has only been observed in fewer than half of treated patients; (2) commercial challenges with costs in the range of $400,000 per patient; (3) supply chain limitations and wait times associated with availability of GMP quality viral vectors, and; (4) long needle-to-needle time from initiation of treatment to receiving the final drug product. Efforts to address some of these challenges using allogeneic CAR T-cell therapies have failed to demonstrate efficacy on par with autologous CAR T-cells. Several safety and regulatory challenges also exist with the use of nucleases to generate allogeneic CAR-T cells, including double strand breaks and the potential for translocation.

RNA Gene Writers leverage target-primed reverse transcription (TPRT) biochemistry evolved from non-LTR retrotransposon mobile genetic elements to modify the genome without the need for DNA breaks. Moreover, RNA Gene Writers can be engineered to catalyze a variety of editing reactions such as substitutions, insertions, and deletions. These edits can be achieved with all-RNA delivery in primary cells and *in vivo*, eliminating the need for viral vectors and DNA template-based gene editing.

When RNA Gene Writers are electroporated into primary human T cells with an RNA template encoding a CAR cassette, we see >20% CAR+ T cells that are viable, proliferative, phenotypically similar (central memory or central effector) to other successful CAR-T products and have a high potential to eradicate tumors *in vitro* through cytotoxicity and cytokine production. Moreover, RNA Gene Writer derived CAR-T cells can be introduced into mouse xenograft models to clear antigen specific tumors *in vivo*, comparable to lentiviral derived CAR-T cells. RNA Gene Writers

can also be packaged into proprietary LNP formulations and delivered to primary human T cells in a mixture of lymphocytes commonly found in patient leukapheresis to generate functional CAR-T cells against tumor cells *in vitro*. This opens the possibility of using RNA Gene Writers to develop a same-day CAR-T treatment. Furthermore, our LNPs deliver RNA to T cells *in vivo*, with 80% reporter expression in a humanized mouse model and 45% in non-human primates (NHP) that may facilitate generation of CAR-T cells *in vivo*.

In addition, the modularity of our RNA Gene Writing technology allows multiplex editing to co-introduce multiple genetic changes via TPRT including generation of universal and more potent CAR-T cells through the knock-out of B2M and TRAC. We show that we can achieve both edits simultaneously in 60% of T cells that are CAR edited.

Our RNA Gene Writing technology is uniquely positioned for one-time delivery of all-RNA components to achieve wide editing capabilities, from gene knock-out to gene integration. This technology will enable simpler manufacturing processes and broader application for immunotherapies. This platform can be used for generating stably integrated CAR T-cells without need for lentivirus or nucleases that could allow for more rapid and cost-effective treatments.

P478

Characterization of plasmacytoid dendritic cell subsets derived from CD34+ hematopoietic stem and progenitor cells

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Plasmacytoid dendritic cells (pDCs) are multifunctional immune cells that have a role in both the innate and adaptive immune system and are known for producing large amounts of type I interferons in response to viral infections. Their functions include cytokine production, antigen presentation, and cytotoxicity, and their potential as an immunotherapy for cancer and infectious disease is being explored. However, broad application of these cells is challenged by their low numbers in the blood representing only 0.1-0.5% of human PBMCs and by their low viability during ex vivo culturing. Our group has previously developed an effective approach for producing pDCs *in vitro* from CD34+ hematopoietic stem and progenitor cells (HSPC-pDCs), which provide an attainable source of pDCs for therapeutic purposes. HSPC-pDCs present pDC characteristics and functions, and like naturally occurring pDCs they exhibit phenotypic heterogeneity based on the expression of the pDC-associated cell surface markers. Here, we decipher this phenotypic variability displayed by HSPC-pDCs by characterizing different subsets. We identify a distinct population of HSPC-pDCs, which are the major producers of IFNα in response to TLR9 stimulation and have an expression profile similar to that of blood-derived pDCs. We also investigated the possibility of rerouting the cell fate of CD34+ HSPC during pDC specification by controlling gene expression of key master transcription factors (TFs). We identify TFs associated with the pDC differentiation trajectory that are essential for the development of IFNα-producing HSPC-pDCs, and we also identify TFs that increase their frequency. In conclusion, we phenotypically and functionally characterize different pDC subsets and modulate their relative frequencies by manipulation of TF expression during differentiation.

P479

Oncolytic measles virus induces proinflammatory functions of tumor-associated myeloid cells

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Attenuated measles virus (MeV) replicates preferentially in tumor cells rather than in healthy cells because they often have defects in the antiviral type I interferon (IFN I) pathway. Our team has shown that in pleural mesothelioma (MPM), 23% of patients have a homozygous deletion of all genes encoding IFN I (IFN-b and IFN-a). They are therefore unable to produce IFN I and are permissive to MeV replication. In other patients, tumor cells produce IFN I but are unresponsive to IFN I. However, non-malignant cells in the tumor microenvironment (TME), particularly tumor-associated macrophages, possess functional antiviral pathways and could produce IFN I upon infection. In this study, we aim to determine the effect of these non-malignant cells the oncolytic activity of MeV and how this might contribute to anti-tumor immunity. We co-cultured MPM cell lines with fibroblasts or monocytes differentiated or not into macrophages and infected them with MeV. Using high-dimensional flow cytometry, we first observed that monocytes co-cultured with tumor cells mainly differenciate into immunosuppressive macrophages. We then show that fibroblasts and myeloid cells inhibit MeV replication in tumor cells with a defect in IFN I production and a functional IFN I receptor signaling. Inhibition of IFN I signaling restored MeV replication in these cells. However, non-malignant cells had no effect on MeV replication in tumor cell lines with a defect in IFN I responsiveness. After co-cultures, we also investigated the transcriptome of sorted tumor and myeloid cells by 3'RNA sequencing and analyzed their secretome and phenotypes. We show that myeloid cells were not permissive for MeV replication. We observed that myeloid cells switched from an immunosuppressive to an immunoactivating phenotype in the presence of infected tumor cells. Upon infection they were able to increase their phagocytic activity, but also the expression of PDL1, costimulatory and HLA molecules, and produced a proinflammatory secretome, in particular IFN I. These IFN I induced the expression of interferon stimulated genes in tumor cells with a defect in IFN I production and protected them from viral replication and lysis. In this study, we show for the first time that tumor-associated macrophages can block MeV oncolytic activity in a patient-specific manner. Overall, our results highlight the importance of considering the non-malignant cells from the TME when assessing the MeV oncolytic activity. They show that non-malignant cells can reduce viral replication in MPM cell lines that respond to IFN I signalling. On the other hand, they also generate a pro-inflammatory microenvironment in response to the virus, which may stimulate the patient's anti-tumor immune response.

P480

Identification of 13 T-cell receptors from SARS-CoV-2-specific CD8+ T-cell clones

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The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a betacoronavirus, is causing the present COVID-19 pandemic.

Several vaccines prevent severe disease and slow down virus spreading, and potential antiviral treatments are under investigation. However, our knowledge about the innate and adaptive immune reactions against the virus, especially about T-cell mediated immunity and protection against reinfection and disease development, still needs to be improved.

SARS-CoV-2 seems to enter the human system without triggering a solid early innate immune response, normally leading to subsequent priming of an adaptive immune response. In the following, the lacking or only slowly developing adaptive immune response that is required to limit the infection allows the virus to cause ongoing cell damage and seems to lead to an overshooting release of cytokines by macrophages and other innate immune cells or even a cytokine storm. In order to investigate T-cell mediated cytokine secretion in SARS-CoV-2 infection, we isolated a library of MHC I-restricted T-cell receptors from SARS-CoV-2-specific CD8+ T-cell clones.

Twenty-one different SARS-CoV-2-specific peptides derived from structural (spike, envelope, membrane, nucleocapsid) and non-structural proteins (nsp3, nsp7, nsp8, ORF3a) were used to stimulate PBMCs of HLA-A2+ donors with either resolved SARS-CoV-2 infection or vaccinated twice against SARS-CoV-2. Following two weeks of in vitro expansion, the cells were re-stimulated with peptide-pulsed T2 cells. Positively stimulated CD8+ cells identified by IFNγ-secretion were single-cell sorted by flow cytometry and clonally expanded. Subsequently, the T-cell receptor sequences of 13 SARS-CoV-2-specific T-cell clones were identified, codon-optimized, cloned into a retroviral vector, and transduced into T-cells from healthy donors for further characterization. The identified T-cell receptors are specific for four different SARS-CoV-2 epitopes from nsp8, ORF3a, and spike protein. To show the recognition of naturally processed peptides, human cell lines HepG2 and A549 stably expressing the respective SARS-CoV-2 proteins were generated via the piggyBac transposon system. In addition, T-cells are co-cultivated with SARS-CoV-2 infected cells to evaluate the antiviral effect of T-cell cytokines.

P481

Overcoming barriers for T cell therapy of chronic hepatitis B virus infection

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Hepatitis B virus (HBV) infection is a severe health problem with 1.5 million new infections per year and 296 million chronically infected patients worldwide, harboring an increased risk to develop liver cirrhosis and hepatocellular carcinoma (HCC). The chronic infection is characterized by an ineffective immune response, with very few, oligoclonal T-cells, showing signs of exhaustion

and dysfunction leading to the persistence of the infection. Hence, a therapeutic vaccination or an adoptive T cell therapy are promising strategies to restore the virus specific T-cell response.

We previously showed that the effectiveness of therapeutic vaccination was diminished in mice replicating HBV at high levels. By administration of small interfering (si)RNAs that knock down all HBV transcripts, the vaccine-induced immune response could be restored, leading to viral clearance. This indicated that high viral antigen levels could prevent induction of an effective immune response against HBV. The aim of this project was to determine whether the effectiveness of pre-activated, redirected T-cells given during T cell therapy would also be influenced by a high HBV antigen load or a large number of infected hepatocytes.

We used T-cells grafted with an HLA-A2 restricted T-cell receptor that is directed against the HBV surface antigen to treat mice persistently infected with HBV. For this purpose, Rag2/Il2rg double knockout mice were infected with AAV-vectors transferring replication competent HBV genomes as well as human HLA-A2 into mouse livers. The impact of the number of target cells was assessed by either high- or low titer rAAV-HBV infections. Mice with high titer infections were additionally treated with siRNA to determine the influence of viral antigen levels. The animals were observed for four weeks, and blood and liver samples were taken to examine viral parameters, HBV-specific immune responses and liver damage.

In this model, the data shows clearance of viral antigens in all groups, indicating that neither a high number of infected hepatocytes nor a high viral antigen load prevent effective antiviral T-cell responses by adoptive T cell therapy. However, a strong increase of liver enzymes indicating hepatocyte damage in mice with high and low titer rAAV-HBV infection but not in siRNA treated mice suggested that reducing the antigen level per cell by siRNA application may be beneficial to reduce T-cell induced toxicity in the liver. In summary, our results indicated that pre-activated, redirected T-cells maintain their antiviral function despite a high antigen load and a high number of infected hepatocytes as target cells.

P482

Universal CAR T cells targeted with HER2-specific soluble linker penetrate spheroids and large tumour xenografts that are inherently resistant to trastuzumab-mediated ADCC

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Cancer therapies based on CAR T cells have made breakthroughs in treating leukaemia but are less effective against solid tumours. An important factor is the monospecific targeting by the CAR, which can make therapy ineffective due to the loss or ab initio absence of the target antigen. Universal CAR T cells (UniCAR Ts) that recognize and bind to target antigens through molecular tag (e.g., biotin) conjugated monoclonal antibodies (linker molecules) might overcome these limitations. In this scenario, sequential or simultaneous administration of various linkers offers the prospect of targeting multiple tumour antigens. We have previously demonstrated that conventional CAR T cells successfully combat ADCC-resistant tumours by actively penetrating their highly developed extracellular matrix (ECM) that presents a barrier to passively diffusing

therapeutic antibodies. This observation raises the question of whether UniCAR Ts, targeted by soluble antibody-derived linkers, can induce antitumor effects in ADCC-resistant tumours where ECM restricts antibody penetration.

To address this question, we have engineered T cells expressing UniCARs that use an affinity-enhanced monomeric streptavidin 2 (mSA2) biotin-binding domain as an extracellular recognition unit that could target HER2 positive tumour cells through biotinylated trastuzumab (BT). We performed conventional immunological assays to investigate *in vitro* activation and cytolytic efficacy of UniCAR T cells in the presence or absence of BT. Recombinant and membrane-bound HER2 molecules served as targets. To explore whether UniCAR Ts can access ECM-masked $HER2^+$ cells through a soluble HER2-targeting linker, we set up a coculture experiment in which MDA-HER2 spheroids were cultured with effector cells for 24h in the presence or absence of biotinylated-trastuzumab. Finally, we compared conventional and universal CAR T cells in a subcutaneous MDA-HER2.ffLUC xenograft model in which UniCAR or HER2-CAR T cells were administered i.v. on day 17 after tumour cell inoculation. Animals treated with UniCAR T cells received 100µg biotinylated trastuzumab i.p. on the day of CAR T cell injection and twice weekly after that.

In activation and coculture assays UniCAR Ts were efficiently activated and induced specific anti-tumor effects in the presence of BT. We also confirmed that activation was proportional to BT concentration. BT-targeted UniCAR Ts penetrated spheroids *in vitro* and induced killing in their core region. *In vivo*, a high level of human $CD4^+$ T cell infiltration was observed in the tumour on day 14 after UniCAR T injection. However, mice that were co-treated with BT and UniCAR Ts died early. This was attributed to infiltration of the lungs by $CD8^+$ UniCAR T cells, which targeted native biotin directly as well as native HER2 through the BT linker.

Our study demonstrates that UniCAR-redirected immune effector cells targeted with soluble linker molecules can be an effective alternative to conventional CAR T cells. These therapeutic systems are well suited for use in patients who have become resistant to antibody therapy and could be particularly beneficial against solid tumours with high antigenic heterogeneity. However, selecting the right recognition domain for the CAR, paired with the appropriate soluble linker, is critical for therapeutic efficacy and safety.

P483

Implementation of TRuC T cells to treat prostate cancer

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Finding new effective treatments for prostate cancer, a leading cause of cancer-related mortality in men, is crucial. Adoptive T cell therapy, like chimeric antigen receptor (CAR) T cell, has shown good results in lymphoid malignancies but proven ineffective against solid tumors, thus far. T cell receptor fusion constructs (TRuCs) comprise a single-chain antibody fragment (scFv) fused to one of the T cell receptor (TCR) subunits. They have been effectively used to repurpose the native TCR to recognize a tumor specific antigen and elicit T cell physiological signaling for effective elimination of the cancer cells. However, TRuC generation currently relies on viral delivery of the transgenic TCR subunit that competes with the corresponding endogenous subunit for TCR assembly. We have developed TRuC T cells recognizing the prostate-specific membrane antigen

(PSMA), a well-characterized target in treating prostate cancer. To improve TRuC T cell engineering, we targeted the integration of a PSMA-specific scFv coding sequence in-frame with the TCR ε-chain gene (*CD3E*). This scar-less scFv knock-in retains the endogenous gene regulation of the ε-chain and simultaneously reduces the competition in TCR assembly, so contributing to the physiological-like features of the TRuC platform. Using an optimized genome-editing protocol based on low dose CRISPR-Cas12a in conjunction with a short DNA fragment coding for the scFv, we achieved viral vector-free knock-in of the PSMA-targeting moiety in 13-34% of T cells isolated from three different donors. CAST-Seq analysis confirmed the high specificity of the *CD3E*-targeting CRISPR-Cas12a nuclease with no detectable off-target effects. *In vitro* functional assays showed comparable cytotoxicity against PSMA-positive cancer cells when comparing canonical CAR T cells to TRuC T cells generated either via the knock-in strategy or viral vector-mediated overexpression of the transgenic TCR subunit fusion. Yet, and importantly, TRuC T cells presented with a subdued pro-inflammatory cytokine-release profile, which is associated with a lower risk of cytokine release syndrome. Upcoming *in vivo* experiments will determine if our revised TRuC T cell platform, as an engineered T cell system closely resembling the native setting, enhances anti-tumor efficiency and safety in a solid tumor model.

P484

A rapid and efficient platform for CAR construct screening

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As the development of Chimeric Antigen Receptor (CAR)-based therapies expands at pace, with notable improvements in efficacy, safety and complexity there is an increased need to streamline the development process. Packaging of CAR plasmids into viral vectors and subsequent primary cell transduction is costly and time consuming, posing a limitation to the number of constructs that can be assessed simultaneously. Generating a non-viral transfection-based platform for CAR screening allows a faster and more cost-effective method for screening multiple CAR constructs. Here, we describe a rapid and efficient non-viral platform for CAR-X development, including a transient, cell line-based platform for screening candidate antigen-binding domains (ABD) and CAR construct assessment utilizing a T cell line (Jurkat). In this system, Jurkat cells may be transfected with CAR-encoding plasmid DNA or mRNA, then co-cultured with antigen positive/negative target cells, and antigen-binding can be evaluated via CD69 and IL-2 expression. This can be used to identify successful CAR binding to target antigens, specificity and also tonic signaling.

Jurkat cells were nucleofected with a HER2 CAR construct and showed robust CAR expression. Following recovery, CAR-Jurkat (CAR-J) were co-cultured overnight with one of the following: HER2- (E:T = 1:1) or HER2+ target cells (E:T = 1:1, 1:0.1 or 1:0.01) and incubated for 24h. Analysis revealed HER2 CAR-J upregulate CD69 in a target specific and E:T dependent manner, with an increase from 5.9% (Her2-) to 30% (HER2+ target cell) demonstrating strong target engagement. We further show this system is flexible and scalable to other constructs.

This platform can be harnessed to screen multiple CAR construct configurations, test target engagement and optimize scFv choice for antigens of interest. It can also reveal CAR-induced

autoactivation, and aid early identification of optimal constructs. Lead CAR(s) can then be taken forward, expressed in primary T cells and functionally evaluated for cellular avidity, potency and ultimately safety. These studies build on a platform to provide end to end support to accelerate the development of new and novel CAR-based cell therapies.

P485

A precision adoptive cell therapy process based on the expansion of clonal neoantigen-reactive T cells from the blood of patients with cancer

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Tumour neoantigens are considered a key determinant in the immune response to cancer. Amongst the different types of neoantigens, clonal neoantigen burden shows the strongest correlation with patient survival and response to immune checkpoint inhibitors pointing to the potential critical role of this target class in cancer immunotherapy.

At Achilles Therapeutics we have developed a clinical protocol for the generation of personalised clonal neoantigen reactive T cells (cNeT) products from a patients' tumour and initiated clinical trials to evaluate their activity against melanoma and non-small cell lung cancer (NSCLC) (NCT03997474, NCT04032847). However, not all cancer patients can undergo surgery or have accessible tumour for generation of cNeT. Importantly, neoantigen-reactive T cells have been identified in the blood of cancer patients pointing to the blood as a potential non-invasive source of clonal neoantigen-reactive cells for therapeutic development. Here, we describe a novel process for the generation of cNeT from the blood of cancer patients and in-depth characterisation supporting the potential therapeutic value of blood-derived cNeT.

Briefly, matched tumour and peripheral blood from patients undergoing routine surgery were obtained from patients through the Achilles Therapeutics trial NCT03517917. Using our proprietary PELEUS™ bioinformatics platform and our NeoRanker immunogenicity tool we generate a list of the most immunogenic clonal neoantigens in a patient's tumour. Immunogenic clonal neoantigens are then included in co-cultures of blood T cells and professional antigen presenting cells to expand cNeT. We perform in-depth characterisation of blood-derived cNeT products to assess their neoantigen-reactive potential using our proprietary potency assay, immunological fitness and clonal composition. T cells were successfully expanded across several solid tumour indications in 37 of 45 patient samples (success rate 82.2%). T cell products had enriched clonal neoantigen reactivity (median 6.4%, range 0.08-81%, n=37). Extensive phenotyping showed that cNeT express markers of activation, cytotoxicity and migration following activation with neoantigen peptides and are capable of killing tumour targets.

Our data demonstrates the feasibility for the expansion of large numbers of cNeT from the blood of cancer patients. Furthermore, product characterisation underscores the fitness and anti-tumour activity of blood-derived cNeT supporting further clinical development of this novel approach.

P486

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 in which we are able to 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.

Building on this platform, we characterized the generated CAR-T cells phenotypically in detail by flow cytometry and supplemented this by CAR copy number variation (CNV) determination via droplet digital (dd)PCR, and analysed CAR surface expression and organization by dSTORM 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, LV/SB CAR-T cells showed higher cytokine production and conferred stronger cytolytic activity compared to KI CAR-T cells in short-term (< 8 hours) lysis assays across multiple effector-to-target (E:T) ratios depending on antigen density. With longer follow-up (> 20 hours) LV/SB CAR-T cells and KI CAR-T cells were equally effective for high and low-antigen

settings. Intriguingly, we observed a significantly lesser extent of activation-induced cell death (AICD) in KI CAR- T-cells, suggesting lower CAR number and density can provide protection from overstimulation. Extended analyses in vitro and pre-clinical in vivo models are 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 to target antigen levels in the respective tumour entity can provide critical advantages for CAR-T cell therapy.

P487

Evaluation of IL23R as a target for CAR-Treg at the site of inflammation in subjects with Crohn's Disease

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Crohn's disease (CD) is a chronic inflammatory bowel disease characterized by uncontrolled immune responses in the gastrointestinal tract. Regulatory T cells (Tregs) play a crucial role in maintaining intestinal homeostasis. However, a local imbalance between Treg and conventional T cell responses contributes to the gut inflammation in CD. Although current treatments can control CD symptoms, treatments that provide long-lasting effects are yet to be developed. By targeting a disease-related protein, Tregs engineered with a chimeric antigen receptor (CAR-Tregs) represent a potential therapeutic alternative for treating CD. We developed a CAR-Treg targeting IL23R. IL23R is overexpressed in CD patients and is an attractive target to activate CAR-Tregs in an inflamed gut. Our IL23R-CAR-Tregs have very low tonic signaling and a desirable signal-to-noise ratio. Moreover, IL23R-CAR-Tregs exhibited IL23R-dependent suppressive activity *in vitro* and in mice with dextran sodium sulfate (DSS)-induced colitis. Here, we show that our IL23R-scFV detects IL23R in intestinal tissues from CD patients. Using biopsy-derived intestinal cells from CD patients, we further characterized the functionality of IL23R-CAR-Tregs. We measured IL23R-specific CAR-Treg activation in response to intestinal cells, mainly in patients with severe CD and, to a lesser extent, with mild-to-moderate CD. Immunohistochemical analysis of intestinal biopsies from moderate-severe CD subjects confirmed that up to 60% of mucosal lamina propria cells express IL23R. Finally, we performed molecular profiling of CD patients to support clinical development. Overall, we confirm that IL23R-CAR-Tregs are specifically activated by IL23R in the inflamed mucosa in CD and provide a promising treatment option for CD patients.

P488

Preclinical evaluation of B7-H3 targeting nanobody-based CAR-T cells in glioblastoma

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Glioblastoma is an aggressive primary brain tumor with a median overall survival of 10 months due to lack of effective therapies. Standard-of-care for glioblastoma patients consists of surgery, radiotherapy and chemotherapy. However, upon relapse, chemoresistance and tumor evasion are observed. The finding that peripheral immune cells can cross the blood-brain-barrier after antigen recognition, provided a rationale for development of immunotherapy for glioblastoma. However, implementation of immunotherapy regimens for glioblastoma patients has been difficult due to its characteristic low tumor mutational burden and defects in antigen-processing and presentation. These features make glioblastoma patients less eligible for immunotherapy approaches depending on the presence of neoantigens and endogenous immune responses. Chimeric antigen receptor (CAR)-T cells, recognize surface-expressed antigens independent of HLA-peptide presentation and represent a promising avenue to treat glioblastoma patients. Classical CARs are designed with single-chain variable fragments (scFv) from monoclonal antibodies, whilst nanobodies derived from camelid heavy chain-only antibodies provide an interesting alternative. Their single-domain nature and high sequence homology with human V_H3 genes avoid the occurrence of tonic signaling, the need for linker-sequence optimization or humanization steps. B7-H3 is considered an ideal target for CAR-T cell therapy of glioblastoma as it is frequently overexpressed in glioblastoma cells. Moreover, expression of B7-H3 on tumor-surrounding healthy brain tissue is not observed. We describe a nanobody-based CAR-T cell therapy targeting B7-H3 in glioblastoma. We incorporated a B7-H3 targeting nanobody in our 2nd generation CAR vector in comparison to a classic scFvCAR incorporating the V_H and V_L sequence of enoblituzumab. We validated the functionality of both CAR constructs using a reporter assay, employing a T cell line expressing green fluorescent protein (GFP) under control of a nuclear factor of activated T cell-dependent promoter. Signaling of the CAR constructs in primary T cells was evaluated based on 4-1BB expression and IFN-γ secretion following antigen recognition. Killing potential was evaluated using a co-culture with GFP$^+$ glioblastoma cells and live cell imaging. Superiority of the nanoCAR construct was observed in these experiments, indicating that classical scFvCAR-T cell therapy can be improved by nanoCAR-T cell therapy. To determine whether nanoCAR-T cells killed tumor cells in an antigen-specific manner, we generated B7-H3 knock-out (KO) cells using CRISPR/Cas9-genome editing and made this KO-variant positive for a red fluorescent protein (RFP). Using GFP$^+$ target versus RFP$^+$ non-target cells, we confirmed the antigen-specific killing of B7-H3$^+$ cells by the nanoCAR-T cells. Altogether, we report development of a promising nanoCAR-T cell product for treatment of glioblastoma. In future, these can be armored with additional factors such as cytokines or immune checkpoint blocking moieties to overcome hurdles in the tumor microenvironment.

P489

Unbiased Comparison of Novel Synthetic Antigen Receptors for T Cells

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Gene editing has the potential to revolutionize adoptive T cell therapies through precise and efficient genetic modifications. Targeted transgene integration of chimeric antigen receptors (CAR) facilitates more predictable T cell function and reduces the risk for insertional mutagenesis. Furthermore, gene editing has enabled the design of entirely novel synthetic antigen receptors, mimicking the T cell receptor (TCR) complex. These TCR-like CAR architectures have been reported to provide higher anti-tumor potency of reprogrammed T cells, particularly when targeting cells with low antigen density. However, there is a lack of unbiased studies that compare the different synthetic antigen receptor architectures regarding their therapeutic potential. Hence,

in this study, we assessed the in vitro and in vivo performance of T cells carrying either classical second-generation CARs containing the CD28 costimulatory domain or TCR-like CAR architectures, such as CD3-epsilon T cell receptor complex fusion constructs (eTruC) or HLA-independent T cell receptors (HIT/STAR).

To generate these receptor architectures, we employed non-viral CRISPR-Cas9 gene editing to generate in-frame fusions of exogenously delivered CD19-specific binders into endogenous genes of the T cell receptor (TCR) complex. We designed distinct templates for knock-in into the *TRAC* gene (for 2nd generation CAR, HIT/STAR), the *CD3-zeta* gene (for 2nd generation CARs), or the *CD3-epsilon* gene (to create eTruC). *In vitro* experiments revealed distinctive characteristics of the receptor designs regarding cytotoxicity and cytokine production. Specifically, T cells redirected with HIT/STAR receptors exhibited superior cytolytic capacity and speed compared to other receptors, resulting in enhanced cytotoxicity toward CD19-positive Nalm-6 leukemia cells and substantial production of IFN-gamma and TNF-alpha. However, in repetitive co-culture experiments, the TCR-like CARs (STAR and eTruC) provided reduced expansion potential compared to T cells redirected with conventional CD19-CARs with a CD28 costimulatory domain. Moreover, after multiple rounds of stimulation, CAR T cells and eTruC T cells exhibited a favorable central memory phenotype, while HIT/STAR receptors demonstrated patterns of exhaustion. Next, we performed a leukemia xenograft mouse model using the CD19-positive Nalm-6 cell line to gain insights into tumor control in vivo. T cells expressing conventional 2nd generation CARs demonstrated superior tumor control in vivo compared to eTruC and HIT/STAR T cells.

This work emphasizes the significance of unbiased side-by-side comparisons of synthetic receptor architectures. We identified performance disparities between the different receptor designs in vitro and in vivo. When targeting tumors with high antigen density, TCR-like CARs provided reduced proliferative capacity and decreased long-term anti-tumor performance in mice. Ongoing studies are designed to further investigate the underlying mechanisms of the suboptimal in vivo performance of TCR-like CAR architectures and devise strategies to overcome them. These findings offer valuable insights for the development of improved immunotherapies targeting tumors and other target cells with gene-edited T cells.

P490

Nanobody-based CD38-specific antibody recruiting molecules: Harnessing polyclonal antibodies for cytotoxicity against myeloma cells

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Antibody recruiting molecules (ARMs) are bispecific immunotherapeutic tools that redirect endogenous antibodies toward tumor cells. ARMs consist of an antibody-binding module and a tumor-binding module, thereby enabling them to recruit polyclonal endogenous antibodies to opsonize target cells. Antibodies recruited to the tumor cell surface can then induce F_c-mediated effector functions such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC), or antibody-dependent cellular phagocytosis (ADCP).

CD38 is overexpressed on multiple myeloma cells and other hematological malignancies. Nanobodies are the single antigen-binding domains derived from camelid heavy-chain antibodies. Their small molecular size and ease of formatting make them ideally suited for the construction of multispecific antibody constructs such as ARMs.

We generated CD38-specific ARMs, consisting of a CD38-specific nanobody fused via a glycine-serine linker either to a nanobody specific for human kappa or lambda light chain (CD38-kappa ARM, CD38-lambda ARM). Our constructs recruit IgG antibodies regardless of their specificity by binding to the kappa light chain (CD38-kappa ARM) or the lambda light chain (CD38-lambda ARM) of human IgG. The CD38-specific nanobody targets the nanobody-based ARMs towards CD38-expressing cancer cells, thus enabling Fc-mediated effector functions.

Both the CD38-kappa ARM and the CD38-lambda ARM bind specifically and simultaneously to CD38-expressing myeloma cells and human immunoglobulin *in vitro*. Both ARMs mediate complement-dependent cytotoxicity against human multiple myeloma cells *in vitro*. Our results demonstrate the potential of nanobody-based CD38-specific ARMs to engage immune effector functions for the killing of myeloma cells.

P491

Whole transcriptome sequencing of *ex vivo* cultured T cells for immunotherapy identifies differential expression of key genes associated with T cell trafficking

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Ex vivo expansion of T cells is a necessary process in the manufacturing of adoptive T cell therapy products. Different expansion conditions and duration of expansion are under investigation to enhance functionality and longer persistence of chimeric antigen receptor (CAR) T cells upon infusion. Recent studies have revealed that CAR T cells accumulate in lungs upon intravenous infusion for up to 72hrs. Meanwhile, it is also shown that CAR T cells glycoengineered to express Sialyl Lewis x (CD15s), exhibit better homing to bone marrow and to other tissues. In order to gain insight into dynamics and functional changes induced in T cells due to *ex vivo* manufacturing conditions, transcriptome-wide changes in *ex vivo* expanded T cells compared to freshly isolated peripheral blood T cells were characterized. Peripheral blood T cells were isolated from healthy male donors aged 20-29 years, using CD3 Fab-Traceless Affinity Cell Selection (TACS) Agarose Columns. Cells were cultured for 7 days in X VIVO 15 media, 5% hAB Serum, 50U IL-2 and were activated using 10µl/ml of TransAct for initial 3 days. Total RNA was isolated from fresh T cells and cells cultured for 4hrs, 12hrs, 24hrs, 3 days and 7 days. TruSeq stranded total RNA libraries were prepared and sequenced on Illumina NovaSeq. Whole transcriptome sequencing and hierarchical clustering of genes identified 27 gene-clusters, with different patterns of transcriptional regulation over time. Gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis for gene-clusters identified significant over-representation of several signaling pathways including mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK), nuclear factor-κB (NF-κB), stress-activated protein kinase (SAPK) and G-protein-coupled receptor (GPCR) signaling pathways in cultured T cells. Genes regulating the DNA replication and cell division bioprocesses are found in gene-cluster 2. Genes involved in DNA transcription and RNA translation machinery are found in gene-clusters 1, 6 and 7. Genes

associated with T cell activation, differentiation, T cell receptor complex and other Immune function-associated genes were clustered in gene-clusters 3, 4 and 11. Integrin and chemokine receptor genes associated with T cell adhesion and migration function were also found in gene-clusters 3 and 4. Integrin gene alpha-6 and chemokine receptor gene CXCR4 were strongly downregulated within 4hrs and then remained nearly constant. Whereas integrin alpha-4 and beta-1 showed gradual 1.5-fold downregulation. Other integrin genes alpha-L, beta-2 and beta-7 showed early downregulation followed by gradual upregulation. CCR6 and CXCR3 showed gradual 4-fold downregulation and gradual 2-fold upregulation respectively. Expression of CCR7 gene remained nearly constant throughout the culture period. This study provides new insights into the early (4hrs) as well as late (7 days) transcriptional changes in T cells during *ex vivo* expansion. Regulation of SAPK and MAPK cascade is indicative of the T cell response to cope with changes in the environment. Meanwhile, the late persistent changes are indicative that *ex vivo* expanded T cells infused as cell therapy products differ from peripheral blood T cells in their trafficking receptor expression. These findings will facilitate investigations on culture conditions to improve adoptive T cell therapy.

P492

Development of Memory Universal CAR T Cells: Overcoming limitations and Enhancing Efficacy in Cancer Therapy

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Cellular therapies based on T cells expressing a chimeric antigen receptor (CAR) represent a breakthrough in cancer treatment, having already demonstrated efficacy in B cell malignancies. However, current therapies based on this strategy rely on autologous products, which involve certain limitations. These primarily stem from the time and cost involved in the manufacturing process, as well as in the poor quality and quantity of T cells in these patients. Moreover, there are additional inconveniences associated with this therapy, particularly related to the variable phenotype of the infused cells.

To address these challenges, our aim was to develop an allogeneic cellular product, which required elimination of B2M and TRAC genes to avoid rejection reactions in patients, but also our goal was to generate a more persistent product, by selecting specific memory cells for the final product. By eliminating the expression of TCR and HLA-I molecules from the surface of our CAR T cells, we expect to avoid both graft versus host and host versus graft reactions in the patient, and with a further isolation step we selected less differentiated T cells to generate a defined product. Finally, we assessed the safety of our strategy, by analyzing the different concerns associated with genome editing.

We successfully generated Memory Universal antiCD19 CAR T cells. The removal of TCR and HLA effectively reduced the bidirectional allogeneic response between cells from different donors while maintaining the lytic capacity of our product. Additionally, Universal CAR T cells with

memory phenotype showed similar lytic activity to conventional CAR-T cells, with better phenotype upon repeated rounds of tumoral encounter.

In summary, we proposed the use of T memory-selected, genome edited CAR T cells, as an improved alternative to standard genome edited CAR-T cells.

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Transactivator-free lentiviral vectors for inducible Advanced Therapy Medicinal Products: application to CAR-T cells

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Externally controlled systems using an inducer are a promising strategy for enhancing the safety and efficacy of gene therapy and immunotherapeutic approaches. However, most inducible ON-systems that become activated by a drug require the presence of a chimeric transcription factor (transactivator), which can be toxic and hinder optimal clinical translation. To overcome this challenge, we previously developed Lent-On-Plus (LOP), the first all-in-one transactivator-free lentiviral vectors (LVs) capable of regulating transgene expression in human stem cells using doxycycline (Dox).

First and foremost, we compared our transactivator-free system, LOP, with a leading competitor that requires a transactivator derived from Herpes Simplex Virus (VP-16) to induce expression, which has been shown to cause different types of cellular toxicities by sequestering transcription factors and altering cell physiology. We comparatively evaluated Dox sensitivity, transgene stability and transcriptional stability as key parameters. Remarkably, our system exhibited 10,000 times more sensitivity to the inducer than our main competitor, and it also demonstrated higher transgene stability over time. Moreover, transcriptional analysis revealed that more than 200 genes were altered in HEK-293T cells transduced with the competitor in the presence of Dox for 7 days, whereas only 7 genes showed altered expression in LOP-transduced cells. These findings highlight the feasibility of using Lent-On-Plus-LV as a potential tool for clinical applications when compared to currently available commercial vectors in terms of safety and robustness.

Next, we evaluated the potential of this LOP vector for generating inducible advanced therapy medicinal products (ATMPs), with a special focus on primary human T cells. In addition, we expanded on this work by generating new versions of the LOP LVs, including different insulators. Our results demonstrated that insertion of the Is2 insulator into the 3′ long terminal repeat of the LOP LVs was essential to control transgene expression in human primary T cells, which was distinct from all other cell types analyzed. Notably, the inducible primary T cells generated by the LOPIs2 LVs were responsive to ultralow doses of Dox (<100 pg/ml) and showed no changes in phenotype or function compared to untransduced T cells. Moreover, the LOPIs2 system effectively regulated transgene expression *in vivo* following oral administration of Dox and reversed to basal levels after Dox withdraw. As a proof-of-concept, we validated the LOPIs2

system by generating inducible CAR-T cells capable of selectively eliminating CD19+ cells in the presence of Dox.

In summary, our findings present the first transactivator-free, all-in-one system capable of generating Dox-inducible ATMPs, offering a safer alternative to currently available TetOn-systems.

P494

New manufacturing strategy for early memory enriched allogeneic NKG2D-CAR T cells: a universal advanced therapy to treat solid tumors

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One of the most promising advances in cancer treatment is Chimeric Antigen Receptor (CAR) T therapy, which has demonstrated impressive clinical results against hematological malignancies. So far, all commercialized therapies are for autologous use and their CARs are only able to recognize one specific cancer antigen. This entails some limitations for their clinical use, including high costs, manufacturing delays, and antigen-negative tumor relapses, among others. In addition, the production strategy is becoming increasingly important as it determines the proportion of memory T cells in the final product, which defines important characteristics for their clinical success, such as *in vivo* CAR-T survival, expansion and long-term persistence.

In order to overcome the aforementioned limitations, we developed a novel allogeneic CAR-T cell therapy by eliminating TCR and HLA class I complexes in healthy donor T cells using CRISPR/Cas9 technology, thus avoiding the risk of graft-versus-host disease and immune rejection, respectively. Furthermore, we lentivirally transduced the atypical NKG2D-CAR, which is based on the ligand-binding domain of NKG2D receptor that targets eight different ligands upregulated in both solid and hematological tumors, thus theoretically being less prone to resistance and tumor relapses. Additionally, we sought to enrich the stem cell memory (scm) T cell subset of our allogeneic NKG2D-CAR T cells by comparing the addition of different interleukin (IL) supplementations (IL-2, IL-7/-15 and IL-7/-15/-21) during culture. CAR T cell products were characterized by flow cytometry and tested for antitumor activity against solid tumor cells by *in vitro* cytotoxicity assays.

Using our manufacturing procedure, we observed a similar TCR/HLA-I negative T cell proportion with the different supplementations but the NKG2D-CAR transduction was significantly more efficient with IL-7/-15 and IL-7/-15/-21 than with IL-2. Regarding the Tscm cell subset, also IL-2 induce a significantly lower percentage than the other supplementations. However, the proliferation of the allogeneic NKG2D-CAR T cells was increased with IL-2 and IL-7/-15/-21 compared to IL-7/-15. All the allogeneic NKG2D-CAR T cell products obtained demonstrated antitumor efficiency against human cervical cancer HeLa cells and colorectal cancer HCT116 and

HT29 cells. Considering all the results, IL-7/-15/-21 supplementation is the most suitable among the IL combinations assayed for the manufacturing of our CAR-T cells and our cell therapy prototype possesses *in vitro* antitumor efficacy against solid tumors. Further research is ongoing to bring this novel allogeneic NKG2D CAR-T cell therapy to clinical application.

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Beneficial effects of induced intra-tumoral cytokine secretion on CAR T cell therapy in solid tumors

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Therapy-resistant solid tumors represent a growing global challenge. For example, neuroblastomas are among the most common malignant solid tumors in children and account for 15% of malignancy-associated childhood deaths. This highlights the urgent need for new therapeutic options. One approach could be immunotherapy, e.g. chimeric antigen receptor (CAR) T cell therapy. CAR T cells show outstanding treatment successes against B cell malignancies but often fail against solid tumors. A major reason for immunotherapy failure in solid tumors is an immunosuppressive tumor microenvironment. Previous studies show that solid tumors with higher immune cell infiltration and cytokine concentrations have a better prognosis and improved susceptibility to immunotherapies. The overarching aim of our group is to develop targeted cytokine gene therapy to overcome the hurdles of an immunosuppressive tumor microenvironment and to facilitate CAR T cell therapy in solid tumors. The approach aims to target the expression of transgenes that encode the T cell-attracting chemokines CXCL10 and CXCL11 or the T cell-stimulating cytokine IFNG to the tumor microenvironment. Their secretion within the tumor will improve CAR T cell infiltration and efficacy, even in tumors with a T cell-excluding signature. So far, we were able to establish a series of transgenic CXCL10-, CXCL11- and IFNG-expressing neuroblastoma cells. After in vitro proof of concept for the targeted transgene knock-in has been obtained in neuroblastoma cell lines the project will now focus on the evaluation of the effector arm of the proposed gene therapeutic approach that is the multi-modal evaluation of the cytokine effects on neuroblastoma directed CAR T cell therapy in vitro and in vivo. We used a 24h IncuCyte® Clearview transwell migration assay (TWMA). Supernatant from the transgenic cell lines containing CXCL10 or CXCL11 could indeed increase CAR T cell migration. This effect was validated in a transendothelial 3D tumor infiltration assay using 3D bioprinted tumors based on the transgenic cell lines. To that end, a 3D bioprinted tumor model had been established in a cooperation with Cellbricks Berlin. Again, increased migration and infiltration of CAR T cells for the chemokine expressing tumor cell lines could be observed. Besides, 2D Co-cultures of transgenic tumor cell lines and neuroblastoma specific CAR T cells are currently being performed to further assess changes in killing behavior, activation and exhaustion as well as T cell phenotype mediated by the different cytokines. Moving forward in vivo with the CXCL10 producing cell lines transplanted to immunodeficient mice we could again show an

increased CAR T cell infiltration in cytokine expressing transgenic tumor cell lines at early time points after infusions compared to the wild type tumors.

To conclude, cytokine secreting neuroblastoma cell lines could be established via targeted knock-in and both in vitro assays and first in vivo tests confirmed improved immunotherapeutic efficiency demonstrating the general feasibility of targeted tumor microenvironment cytokine gene therapy in neuroblastoma.

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NOn-Viral gene-modified STEM cell therapy (NOVISTEM)

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The successful implementation of cell and gene therapies at a high scale comes with several challenges, including product manufacturing time, availability of transfection reagents, cost-effectiveness, and risk of therapy-associated adverse events. The main objective of our European Innovation Council-funded NOVISTEM project is to establish an induced pluripotent stem cell (iPSC)-based cell and gene therapy platform in which iPSCs are genetically engineered by a novel gentle transfection system and subsequently differentiated by a streamlined *in vitro* protocol to generate autologous gene-corrected blood stem cells or allogeneic chimeric antigen receptor (CAR)-T cells. Compared to traditional autologous hematopoietic stem cell (HSC) transplantation therapies, this iPSC-based strategy has the benefit of easier cell expansion and more efficient gene-editing, enabling the unlimited generation of healthy HSCs. These advantages apply for iPSC derived CAR-T therapy as well, since current *ex vivo* culturing and gene-editing of mature T cells results in terminal T cell differentiation and increased cell exhaustion. In addition, indefinite availability of healthy donor iPSC cell lines permits the use of a superior starting material compared to the limited quantity and often poor quality of T cells derived from patients.

Specifically, we make use of photoporation as a next-generation transfection technology combining high delivery efficiencies with excellent cell viability. Importantly, in comparison to state-of-the-art commercially available physical transfection platforms, photoporation minimally affects cellular homeostasis and proliferation, thus maximizing the cell's therapeutic potential. Here, we aim to further optimize photoporation for site-specific CRISPR-mediated genetic engineering of iPSCs. Next, via optimisation of an *in vitro* differentiation system, the genetically modified iPSCs are differentiated towards CAR-T cells or gene-corrected blood forming cells. For example, we activate TNF receptor 2 to enhance differentiation of CAR-engineered iPSCs into T cell precursors which in turn will mature as CAR-expressing T cells. The aim is to pave the road towards clinical implementation for which a high-throughput photoporation platform will be built. This work could beneficially impact both the accessibility and production of autologous and allogeneic cell therapy products, as well as establish a platform for further academic and clinical research within the (hematopoietic) stem cell field.

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Expansion and T cell phenotype can be tuned in stirred tank bioreactors through temporal control of T cell activation stimuli

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Adoptive cell therapies using chimeric antigen receptor (CAR) T cells have shown great promise in treating cancer. Still, while CAR T cells addressing haematological malignancies have achieved encouraging results, no equivalent successes have been observed in the context of solid tumours, in part due to their suboptimal persistence *in vivo*. The ability of T cells to engraft following their infusion in the patient is related to their state of differentiation as products with a high number of naïve and central memory T cells have shown greatest potency in several preclinical studies. Given that the rate of T cell proliferation/differentiation and their potency attributes are largely determined during T cell activation, precise control over initiation and termination of the activation stimuli is critical to maximize cell expansion while controlling T cell phenotype and function. Besides, the environmental factors that govern the heterogeneity of T cells generated *in vitro* are still largely unknown alongside their impact on the cell's potency, existing the need for increased control over cell culture parameters to maximize the production of high-quality T cell subsets.

To circumvent the current bottlenecks regarding poor control and downstream removal of gold standard activation strategies to terminate the activation step (Dynabeads™ and TransAct™), we propose to develop an integrated manufacturing process in stirred tank bioreactors (STB) for controlled activation and production of T cells using microbeads functionalized with anti-CD3/CD28 antibodies. We envision that the distinct size and density of T cells and of the microbeads could be explored to achieve optimal kinetics of contact between them, providing enhanced control over the T cell activation and expansion steps and, consequently, over the cell phenotype, avoiding their exhaustion.

We demonstrated the microbeads' ability to efficiently activate T cells under agitation conditions, as assessed by the expression of T cell activation markers CD25 and CD69. A design of experiment (DOE) was performed to determine an optimal ratio of cells/microbeads, as well as the time of activation and further expansion to produce more potent cellular phenotypes. The potential of the applied strategy is further highlighted by the reduced expression of T cell exhaustion markers PD1 and CD57 compared to the control condition exploring Dynabeads™ or TransAct™ to activate T cells. Key process parameters (e.g. stirring speed and profile, feeding regimen, medium composition) were optimized to improve T cell expansion in STB (Ambr® 15 Microbioreactor System) without compromising their quality attributes. Total cell numbers and T cell subpopulations regarding their naïve (CD45RA+CCR7+), central memory (CCR7+CD45RA-) and effector memory (CCR7-CD45RA-) phenotype were analysed by flow cytometry.

The optimized activation and expansion strategies implemented in STB led to reduced expression of exhaustion markers by over 20%, while favoring the manufacture of naïve and central memory T cells, providing evidence of its potential for the scalable production of high-quality T cells.

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Comparative evaluation of CAR-NK and CAR-T cell therapy for HNSCC: a novel approach with enhanced accessibility and safety

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Chimeric antigen receptor T (CAR-T) cell therapy has revolutionised the treatment of haematological malignancies, yielding remarkable clinical outcomes. However, its efficacy in solid tumors remains limited due to challenges in tumor penetration. To overcome this constraint, CAR natural killer (CAR-NK) cell therapy has emerged as a promising alternative, offering improved tumor accessibility, enhanced safety, and convenient off-the-shelf manufacturing. In this study, our objective is to develop a novel cellular therapy for Head and Neck Squamous Cell Cancer (HNSCC) based on CAR-NK and compare its efficacy with the established CAR-T approach.

We employed a bioinformatic pipeline to identify targetable tumor-associated antigens by leveraging cancer and genomic databases, resulting in the identification of twelve protein candidates classified into three tiers. Through real-time PCR (qPCR), we evaluated the expression levels of these candidates in various HNSCC cell lines, ultimately prioritising two promising targets: a transmembrane adhesion receptor and a GPI-anchored cell membrane receptor. Employing *in silico* techniques, we designed and assessed several candidate single chain variable fragments (scFvs) directed towards these target proteins. Subsequently, we generated second-generation CAR-NK and CAR-T cells, incorporating 4-1BB and CD3z costimulatory domains, via lentiviral transfection of NK-92 and Jurkat cell lines with plasmids containing the CAR constructs. Through co-culture experiments, we evaluated the *in vitro* cytotoxic activity of these modified cells by assessing the activation and viability markers via flow cytometry analysis to determine which edited cells demonstrated superior results.

Our next step will be to modify the CAR constructs by incorporating a NKG2D transmembrane domain and 2B4-CD3z costimulatory domains typically found in NK cells. This modification aims to explore whether CAR-NK cells transduced with this enhanced CAR configuration exhibit increased activity and potency.

This study underscores the potential of CAR-NK cell therapy as a promising avenue for solid tumor treatment, specifically in HNSCC. By capitalising on CAR-NK cells' superior tumor accessibility, improved safety profile, and readily available manufacturing, we aim to address the existing limitations of CAR-T therapy. Our findings will shed light on the comparative efficacy of CAR-NK and CAR-T approaches, paving the way for future advancements in cellular therapies against solid tumors.

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AURKA-inhibitor resistant CAR T cells in combination with AURKAi for advanced neuroblastoma cell killing

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Neuroblastoma is the most common extracranial solid tumor in children, accounting for 15% of all pediatric cancer deaths worldwide. The main oncogenic driver of neuroblastoma is MYCN, which is always associated with a high-risk disease and therapy resistance. Conventional therapies are not effective for over 50% of high-risk neuroblastoma patients thus personalized therapies are urgently needed. So far, attempts to target MYCN directly have failed due to the unavailability of obvious binding pockets for small molecule inhibitors. Thus, Aurora A kinase inhibitors (AURKAi), such as the highly effective AURKAi LY3295668, are becoming interesting candidates to indirectly inhibit MYCN activity by causing its proteasomal degradation. GD2-CAR T cells have only recently shown clinical success, highlighting the capacity to further improve neuroblastoma-directed immunotherapies. Here, we aim to improve neuroblastoma cell killing by combining the AURKAi LY3295668 with neuroblastoma-specific CAR T cells targeting L1CAM. Previously, we demonstrated that AURKA function is necessary for proper proliferation and effector function of CAR T cells. Hence, we created CAR T cells overexpressing the AURKA T217A mutant, which is predicted to confer resistance against AURKAi LY3295668, thus preventing reduced CAR T cell function in the presence of the inhibitor. We are currently confirming the resistance of L1CAM CAR T cells harboring the mutant AURKA transgene to AURKAi LY3295668. Next, we will look at L1CAM CAR T cells harboring either the wild type or mutant AURKA in in vitro coculture experiments for their effector function against neuroblastoma cell lines in combination with AURKAi LY3295668. In these experiments, neuroblastoma cell lines with (IMR5/75, SK-N-BE(2), Kelly) or without MYCN amplification (SK-N-SH, SK-N-AS) are cocultured with CAR T cells either with or without the AURKAi LY3295668. CAR T cell function is assessed by their release of effector cytokines (IFNG and IL2), expression of markers of activation (CD69, CD25, CD137) and exhaustion (LAG3, TIM3, PD-1) and their cytotoxic potential using the Incucyte live cell analysis instrument to detect tumor cell killing. The data will show whether the combination of neuroblastoma-specific CAR T cells equipped with AURKA T217A mutant and pharmacological inhibition of MYCN can enhance killing efficacy against MYCN-amplified neuroblastoma cell lines and improve therapeutic outcome for patients in the clinic.

P500

CD28 and 41BB Costimulatory Domains Alone or in Combination Differentially Influence Cell Surface Dynamics and Organization of Chimeric Antigen Receptors and Early Activation of CAR T Cells

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After more than three decades of development, chimeric antigen receptor (CAR) modified T cell therapy has emerged as a promising strategy for cancer immunotherapy. Clinically, CD19-targeting second-generation CAR T cells have represented a paradigm shift in treating chemotherapy-resistant leukemias and lymphomas. However, the clinical experience with CAR T cells in solid tumor trials has been disappointing. Although CAR T cell therapy could be an encouraging approach in solid tumor therapy, ineffectiveness and safety issues are becoming increasingly apparent, suggesting that their further development requires molecular-level optimization. While new information is continually surfacing on the molecular level functioning of TCR-based synapses, we still do not completely understand how CAR molecules and CAR-derived immune synapses are organized and function at the submicron level.

In the present study, we performed a systematic, comprehensive analysis of the molecular organization in the T cell membrane of first-, second-, and third-generation HER2-specific CARs encompassing CD28 and/or 41BB costimulation. We studied the antigen-independent self-association and membrane diffusion kinetics of first- (.z), second- (CD28.z, 41BB.z), and third-(CD28.41BB.z) generation HER2-specific CARs in the resting T cell membrane using super-resolution AiryScan microscopy and fluorescence correlation spectroscopy, in correlation with RoseTTAFold-based structure prediction and assessment of oligomerization in native Western blot.

We found while .z and CD28.z CARs formed large, high-density submicron clusters of dimers, 41BB-containing CARs formed higher oligomers that assembled into smaller but more numerous membrane clusters. The first-, second- and third-generation CARs showed progressively increasing lateral diffusion as the distance of their CD3z domain from the membrane plane increased. Confocal microscopy analysis of immunological synapses showed that both small but highly mobile CD28.41BB.z and large but less mobile .z CAR clusters induced more efficient CD3ζ and pLck phosphorylation than CD28.z or 41BB.z CARs organized in clusters of intermediate size and mobility. However, electric cell-substrate impedance sensing revealed that the CD28.41BB.z CAR performs worst in sequential short-term elimination of adherent tumor cells, while the .z CAR is superior to all others.

Our results suggest that although first-generation HER2.z CARs form fewer, large, high-density immobile clusters in the cell membrane than third-generation HER2.CD28.41BB. z CARs, which are assembled into a large number of small, fragmented, mobile clusters, both of these combinations of dynamics and topology were highly effective in forming active immune synapses, compared to second-generation CARs, which showed intermediate mobility and cluster size and were less active in immune synapses. In parallel, we found that the diverse membrane organization and mobility of CARs containing different costimulations are consistent with their modeled molecular structure and have functional consequences in that HER2.z CAR T cells bearing large, high-density, preformed clusters are the most efficacious during the first day of the tumor cell killing in vitro.

We conclude that the molecular structure, membrane organization, and mobility of CARs are critical design parameters that can predict the development of an effective immune synapse. Therefore, they need to be considered alongside the long-term biological effects of costimulatory domains to achieve an optimal therapeutic effect.

P501

pdTRUCKIL-15 CAR-T cells expressing IL-15 under PD-1 endogenous promoter show an improved phenotype and potency of CAR-T cell therapy

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Adoptive cell therapy (ACT) with genetically engineered T cells expressing chimeric antigen receptors (CARs) has emerged as a promising treatment option for refractory leukaemia or lymphoma patients. Despite its success in type B malignancies, CAR-T cell therapy still faces challenges such as toxicity, inactivation by the tumour microenvironment (TME), and low cell persistence in patients. In this study we aim to investigate the potential use of gene editing (GE) using the CRISPR/Cas9 system to repurpose the PD-1 locus, which is a crucial component of the T cell activation-suppression pathway and to express in this locus IL-15, promoting this way an improved phenotype and therapy potency.

The goal is to achieve a fourth generation CAR-T cells enhancing the effectiveness and longevity of these CAR-T cells by inserting IL-15 within PD-1 locus. To achieve these new CARs, we will use the nuclease CRISPR/Cas9 like GE tool together with AAV6 containing the template of IL-15, without exogenous promoter, as Donor delivery tool. At end point, we will achieve a fourth generation CAR with a controlled expression of IL-15 under PD-1 endogenous promoter which are called pdTRUCKIL-15 CAR-T cells. To achieve this Knock-In (KI) experiments, we have studied the possibility to use ssDNA like Donor delivery tool like a safety method in comparison with AAV6 (viral methods) but we decided to continue using AAV6 due to their high KI efficacy rates (average of 50% of site-specific integrations).

In this study we evaluated the impact of PD-1 KO and the IL-15 controlled expression on the phenotype, proliferation, respiratory capacity, anti-apoptotic proteins, and lytic activity of newly generated CAR-T cells.

CAR-T cells lacking PD-1 exhibited a decreased expansion capacity and fitness despite to present an improvement in lysis capacity of PDL1+ target cells. However, when IL-15 was Knocked-In in this locus, it resulted in CAR-T cells PD-1 KO with an improved phenotype, viability, and overall cell wellness together with an improved lytic capacity showing more persistence on killing assays and an improved specific lysis in comparison with CAR-T cells PD1 KO.

Furthermore, when these pdTRUCKIL-15 T cells were activated and expressed IL15 under PD1 endogenous promoter, exhibited a significantly decreased expression of pro-apoptotic proteins like BIM and a significant increased anti-apoptotic proteins expression like Bcl-2 and Bcl-xL showing an increased resistance to apoptosis.

P502

Optimization of CAR design, transduction, and CAR T cell expansion for the treatment of colorectal cancer

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CAR T cell therapy is approved for some hematological tumors, like B cell acute lymphoblastic leukemia and diffuse large B cell lymphoma. This novel form of gene therapy resulted in remarkable clinical outcomes. Thus, there is a growing interest in exploring CAR T cell therapy for solid tumors including colorectal cancer (CRC). One of the major challenges in developing CRC-specific CARs is the lack of uniformly expressed and tumor-specific antigens. In an unbiased approach to identify novel CRC-specific antigens, the monoclonal antibody (mAb) 20G4 was produced and tested by high-throughput flow cytometric screening (FACS) for the specificity in the recognition of CRC cells and CRC stem cells (CRC-SCs), the emerging tumor cell subpopulations that can drive tumor growth and are often responsible for the resistance to conventional therapy. The first aim of this project was to evaluate the selective binding capacity of 20G4 mAb on tumor cells. We found that it preferentially bound to the surface of the CRC-SC when compared to the commercial lines. Moreover, using the scFv sequence information of the 20G4 tumor-specific mAb, a novel CAR construct targeting the human CRC-SCs was designed, and the conditions for the generation and the expansion of 20G4-CAR T cells were established. In this part of the study, we optimized the preparation of recombinant lentivirus and CAR T cells by focusing on construct optimization, promoter choice, and transduction systems. T cells from peripheral blood of healthy donors were transduced with a third-generation lentiviral vector carrying a second-generation CAR molecule driven by different promoters and a fluorescent reporter gene. These CAR T cells were subsequently tested for their cytotoxic activity against target antigen-expressing CRC-SCs in vitro. We observed that CAR T transduced with EF-1 promoter exhibited the best killing ability and they secreted pro-inflammatory cytokines (IL-2, IFN-γ) confirming their functionality and antigen specificity. To optimize the efficiency of CAR T cell expansion, we investigated the use of common γ-chain cytokines IL-7 and IL-15 over a 20-day period. When compared to the culture with IL-2 alone, this culture method resulted in superior T cell expansion and activation. The findings of this study proved the feasibility to generate high numbers of tumoricidal 20G4 CAR T cells in vitro and paved the way for further preclinical studies to determine their in vivo preclinical efficacy in murine models.

P503

Multimerization of Chimeric Antigen Receptor (CAR) binding domains: A solution to assess tissue specificity of low to medium affinity scFv

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Off-target binding of therapeutic antibodies to non-target tissues can result in adverse events (AE). Non-specific binding of a scFv (binding moiety) in the context of a CAR therapy can be considered even more detrimental due to potential AEs like cytokine release syndrome (CRS) and lack of efficacy. Hence, careful assessment of CAR specificity is imperative prior to clinical application by determining the on- and off-target binding capabilities in different tissues. ScFv affinities for various targets vary between low to medium, making this assessment challenging when using standard immunohistochemical (IHC) protocols. In the past high-affinity surrogate antibodies have been used to circumvent this problem, which is time-consuming and requires comparability studies. Here we describe an avidity enhancement approach to allow rapid, sensitive detection and characterization of the CAR without the need for extensive comparability studies.

Increasing the avidity of a molecule for its target means increasing its binding capacity while preserving the binding specificity. We generated and evaluated the binding properties of either a dimerized, Fc-tagged based scFv, or a multimeric scFv bound to a dextran backbone. By applying this technique to Sangamo's preclinical aIL23R-CAR (see respective Sangamo abstract), we established a highly sensitive, specific detection method for IL23R in flow cytometry and histology.
In conclusion, avidity-enhanced reagents provide a rapid method to validate in-vivo targets and allow cell tracking. Further, they allow the detection of potential off-target binding for CARs in development.

P504

Quantification of single cell vector copy number in CAR T cell products utilizing a novel microfluidic technology

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Cell and gene therapies are altering the treatment landscape of intractable genetic disorders, including cancer and inherited diseases. One of the transformative solutions to treat cancer patients has been the Chimeric antigen receptor T-Cell (CAR-T) immunotherapy. As most CAR-T therapies rely on the modification of host cells with CAR using lentiviral vectors, followed by the re-introduction of the modified T-cell into the patient, the quality of these modified cells is extensively regulated to warrant safety. Accurate measurement of gene transfer as well as viral vector copy number (VCN) is critical to therapeutic development and a key attribute for assessing safety and efficacy. Conventional methods measuring VCN report out population average (bulk) or

involve laborious and time-consuming single-cell clonal outgrowth procedures which could take up to months to complete.

Here, we introduce a novel microfluidic workflow to precisely measure vector copy number as well as transduction efficiency across thousands of cells at a single-cell resolution in a shorter time frame. The technology is enabled by a two-droplet system. The first droplet encapsulates thousands of cells individually followed by lysis of the cells, releasing DNA from its heterochromatin state for uniform DNA interrogation. The second droplet generation involves uniquely barcoding individual lysates and amplifying the target DNA via multiplexed PCR inside each droplet. The targets are processed using NGS library preparation methods and the final products are sequenced on an NGS sequencing instrument. In this study, the single-cell VCN data generated using our vector-specific targeted assays was orthogonally validated by ddPCR single assays to obtain a correlation of 99%. Using vector-specific amplicons to classify cells as transduced or non-transduced allows us to calculate the percent transduction for each sample. Using admixtures of transduced and non-transduced cell lines, we show our % Transduction assay has >98.8% and >99.2% specificity and sensitivity.

P505

Advancing precision genome editing and safety in cellular immunotherapy

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Chimeric antigen receptor T cell therapy (CAR -T) has shown remarkable clinical success in the treatment of hematologic malignancies. However, there is still a need to improve the control and safety of CAR -T cell activity. Small molecules represent an attractive strategy to regulate CAR -T cell function and offer a versatile approach to improve the precision and safety of immunotherapy. A commonly used approach is the incorporation of small molecule-induced degraders, such as proteolysis targeting chimeras (PROTACs) or small molecule-based degron systems, into CAR T cells. These small molecules selectively recruit E3 ubiquitin ligases to target and degrade specific proteins in CAR T cells. By selecting target proteins that are critical to CAR T cell function, such as CAR molecules themselves or essential signaling components, the inducible degradation system can effectively eliminate CAR T cells following small molecule administration.
While currently approved therapy involves relatively effective lentiviral delivery of CAR to T cells, the use of CRISPR/Cas9 for target integration offers several advantages. The CRISPR-Cas system enables simultaneous editing of endogenous T cell receptor (TCR) genes or precise editing of key immune-related genes in CAR T cells. This includes altering genes involved in T cell exhaustion, regulation of immune checkpoints, and secretion of cytokines, among others. By altering these genes, CAR T cells can be modified to resist immunosuppressive signals in the tumor microenvironment, promote persistence, and enhance antitumor activity.
While lentiviral delivery integrates CAR receptors at a random site into the genome, the CRISPR/Cas9 system allows targeted integration at specific sites. However, there is room for improvement in target delivery of larger gene fragments by armoring a well-established CRISPR/Cas9 system with additional enzyme that allow either more efficient or safer integration of gene fragments into the genome.

We designed CAR receptors with molecular ON - and OFF switches by adding an additional domain that can respond to exogenous delivery of small molecules. We chose the regulatory

domain that has three key features: i) the protein domain is of fully human origin, ii) it can be regulated by FDA-approved small molecules, and iii) it does not respond to the physiological level of natural ligand. We demonstrated that expression of CAR receptors, and thus tumor cell killing and cytokine secretion, are concentration-dependent on the presence of the ligand.

Inducible degradation systems are a promising approach to regulate the persistence and activity of CAR T cells and provide a means to improve the precision and safety of cellular immunotherapy. At the same time, further exploration and development of target integration strategies will undoubtedly contribute to the advancement of CAR T-cell therapy and its broader application in the treatment of cancer and other diseases.

P506

Development of a manufacturing platform for umbilical cord blood natural killer cells and derived extracellular vesicles for immunotherapy

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Umbilical cord blood (UCB) natural killer (NK) cell-based immunotherapy is a promising approach for treating various malignancies, including blood cancers. UCB is an attractive source of NK cells due to its allogeneic nature, low immunogenicity, and easy availability. Another potential treatment strategy for these blood cancers involves using extracellular vesicles (EVs) naturally secreted by different cell types, including NK cells, as a cell-free immunotherapy option. However, obtaining sufficient functional UCB-NK cells and their derived EVs is challenging. This study aims to address this challenge by investigating the expansion of UCB-NK cells using different culture media and evaluating their impact on the NK cell population. Enriched CD56+ cells from UCB samples were cultured in various media supplemented with interleukin-2, including X-VIVO 15, NK MACS, CTS NK-Xpander, StemSpan SFEM II, PRIME-XV NK Cell CDM, and GMP SCGM. Flow cytometry analysis was performed at different time points and the expansion fold of total cells and NK cells, as well as NK cell percentage, were calculated. The activation status using additional markers, as well as CD107a degranulation and cytotoxicity of NK cells against the K562 cell line were assessed. Among the tested media, NK MACS with 2% supplement showed the most favourable condition for expanding cells. NK MACS with 1% supplement and CTS NK-Xpander followed, with similar fold increases. In contrast, StemSpan SFEM II and GMP SCGM resulted in reduced cell expansion, while X-VIVO 15 and PRIME-XV NK Cell CDM were ineffective. All conditions maintained a CD3-CD56+ NK cell percentage above 90% without significant T cell contamination. The expansion process improved the activation state of NK cells, as indicated by increased degranulation and cytotoxicity against the K562 cell line. Following NK cell expansion, a conditioning phase was conducted by replacing the expansion medium with basal medium without human serum or with exosome-depleted human platelet lysate. The optimal time point for EV collection was determined and the harvested cells were characterized after conditioning. The cell culture supernatant containing EVs was clarified by microfiltration, concentrated and diafiltrated. DNA digestion was performed and EVs were isolated by anion-exchange chromatography. Results showed that cells exhibited a significantly higher particle production when exosome-depleted human platelet lysate was used. These cells also exhibited consistent viability and could be cultured for an additional 48 hours of conditioning. Currently, NK-EV and

impurities populations are being characterized using techniques such as nanoparticle tracking analysis, protein quantification, western blot and imaging. The morphology and structure of the manufactured EVs are being assessed by transmission electron microscopy and cryogenic electron microscopy. Finally, NK-derived EVs are being included in the cytotoxicity assays. Future work will include the study of the impact of culture parameters, such as O_2 concentration, on EV production. The data obtained herein is critical for determining the optimal conditions for producing well-characterized NK cells-derived EVs, which will pave the way for the development of robust manufacturing processes.

P507

Towards smart manufacturing for autologous cell therapies enabled by innovative biomonitoring technologies and advanced process control

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Autologous immunotherapies have achieved impressive treatment results in patients with blood cancers. The next-generation of personalised immunotherapies include tumour-infiltrating lymphocytes (TIL) to overcome the limitations of adaptive therapy, including CAR-T modalities, in the treatment of solid tumours. Lack of effective, fast, adaptive, controllable, and scalable manufacturing processes remains one of the critical bottlenecks for clinical adoption of such complex personalised cell therapies. The EU-funded SMARTER consortium is led by Achilles Therapeutics UK Limited, a clinical-stage company developing autologous cell therapies, in partnership with the Cell and Gene Therapy Catapult, and academic experts in process biomarker discovery at Instituto de Investigacion Sanitaria La Fe and in bioprocess sensor development at Leibniz Universitat Hannover. Together, the consortium aims to develop a first-in-class, smart bioprocessing manufacturing platform for personalised autologous cell therapies, implementing for the first time in-line process analytical technologies and advanced process control systems. The project exploits breakthrough discoveries of novel T cell expansion process biomarkers and development of new fluorescence spectroscopy sensors for real-time monitoring of critical process parameters, to enable adaptive process control of the precision TIL biomanufacturing process. We describe here an overview of the approach that has been undertaken to achieve the goals within the first 12 months of the consortium project. Briefly, a surrogate healthy-donor model was developed to generate tumour infiltrating lymphocyte (TIL)-like T cells that more closely represent TILs than standard healthy-donor derived cells. The model was utilised to support process transfer to consortium partners. Concurrent metabolite profiling was performed to identify conserved and divergent biomarkers, which shall putatively include targets for future advanced process control development.

P508

Adapter CAR platform demonstrates potential to improve safety and immunophenotype of CAR T cells

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Chimeric antigen receptor (CAR) T cell therapy has emerged as a powerful approach to treat hematological cancer. However, safety concerns related to the inability to control CAR T cell responses in patients remain a significant challenge.

The Adapter CAR™ (AdCAR) system has been developed as a versatile and tunable platform, in which AdCARs are redirected to surface antigens via adapter molecules (AM). AdCARs recognize a neoepitope-like structure, referred to as linker-label epitope (LLE).

Here, both platforms, AdCAR and conventional CAR, were compared in a 3rd generation backbone including CD28.4-1BB co-stimulatory domains. AdCAR T cells were redirected against the acute myeloid leukemia cell line OCI-AML2 using a CD33-targeted AM, whereas conventional anti-CD33 CAR T cells targeted the tumor cells directly. AdCAR T cells showed delayed, but complete, tumor cell lysis accompanied by a less differentiated and exhausted immunophenotype. The response of AdCAR T cells was scalable by addition of different AM doses. In absence of AM no background activation of AdCAR T cells was observed highlighting the controllability of the AdCAR platform.

Based on these results, the AdCAR T cell platform provides improved flexibility and favorable T cell immunophenotype compared to conventional CAR T cells, offering a potential for a safer and more effective CAR T cell therapy.

P509

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

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Mucosal-dominant pemphigus vulgaris (mPV) is a painful autoimmune blistering 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 are evaluating the safety and activity of a novel cellular therapy consisting of gene-modified autologous T cells (DSG3-CAART) engineered to eliminate DSG3 reactive B cells in mPV patients (NCT04422912). We have previously reported that DSG3-CAART cells were detected in the blood of all mPV subjects post-infusion without preconditioning ($2x10^7$ –

$7.5x10^9$ transduced CAART cells per dose). A dose-dependent increase in peak persistence (C_{max}) and persistence AUC for the first 29 days (AUC_{29d}) resulted from escalating doses of $2x10^7$ to $2.5x10^9$ DSG3-CAART cells. Doses exceeding $2.5x10^9$ DSG3-CAART cells (up to $7.5x10^9$) failed to further increase persistence. We examined an additional patient cohort utilizing a preconditioning regimen consisting of intravenous immune globulin (IVIG) to reduce potentially neutralizing autoantibodies and cyclophosphamide (Cy) to reduce leukocytes, followed by an infusion of $2.5x10^9$ DSG3-CAART cells (n=3). Further, we examined patients receiving two doses of $5\text{-}7.5x10^9$ DSG3-CAART cells administered 21 days apart to extend CAART exposure (n=2). Here, we report on data from subjects in these additional cohorts and their impact on CAART cell persistence. Both persistence C_{max} and AUC_{29d} were unchanged in subjects receiving preconditioning therapy with IVIG + Cy compared to subjects receiving DSG3-CAART alone. In subjects receiving preconditioning with IVIG + Cy, transient (< 2 weeks) leukopenia and neutropenia were observed without lymphopenia in parallel with a transient and modest increase in serum IL-15 concentration compared to subjects without preconditioning. DSG3-CAART cell activation post-infusion was not impacted by the IVIG + Cy preconditioning regimen. For the one patient analyzed so far that received multiple doses of DSG3-CAART cells, persistence was increased compared to subjects receiving a single dose of DSG3-CART cells. To date, there is no clear pattern of change in anti-DSG3 autoantibody levels across dosing cohorts despite increases in DSG3-CAART cell exposure. These data support continued exploration of DSG3-CAART with more aggressive preconditioning regimens including the addition of fludarabine for mPV patients.

P510

Shifted into second gear: Biparatopic CD38-specific nanobody-based CAR-NK cells for treatment of multiple myeloma

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Nanobodies are the antigen-binding single variable immunoglobulin domains of camelid heavy-chain antibodies. The key advantage of nanobodies is improved solubility compared to the corresponding pair of variable (V) domains of a conventional antibody. Nanobodies do not exhibit the pairing issues seen with the pair of variable domains of a conventional antibody (single-chain variable fragment, scFv). Their singular binding domain format allows for easy reformatting into mono-, bi-, and multispecific proteins in a LEGO-brick-like fashion. These features make nanobodies particularly suitable as binding domains for chimeric antigen receptors (CARs). Cilta-Cel (Ciltacabtagene Autoleucel), a nanobody-based BCMA-specific CAR therapy for multiple myeloma, has received FDA and EMA approval in light of its excellent clinical results. The binding domain of Cilta-Cel is composed of two genetically linked BCMA-specific nanobodies targeting non-overlapping epitopes on BCMA. Remarkably, 10-fold lower numbers of allogenic T cells expressing Cilta-Cel are needed to achieve complete remission compared to most scFv-based CARs.

CD38 is a type II membrane protein that is overexpressed by multiple myeloma cells. Our group has previously cloned and expressed CD38-specific nanobody-based CARs in NK92 cells. These nanobody-based CAR-NK cells showed potent cytotoxicity against CD38-expressing cell lines *in vitro* and against primary multiple myeloma cells *ex vivo*. To evaluate if a biparatopic targeting strategy can improve cytotoxicity against myeloma cells, we have now generated CD38-specific

biparatopic nanobody-based CAR-NK cells. These show binding to CD38 and lysis of CD38-expressing cells *in vitro*. These results demonstrate that functional biparatopic CAR-NK cells can be generated using CD38-specific nanobodies.

Biparatopic CD38-specific nanobody-based CAR NK cells therefore hold promise as new 'off-the-shelf' therapeutic tools for the therapy of multiple myeloma.

P511

Using iPSC and gene editing technologies to generate immune-cloaked "off-the-shelf' cell therapies

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Human induced pluripotent stem cells (iPSCs) can be renewed, genetically modified, scaled and differentiated into almost any cell type. These characteristics make iPSCs an excellent tool for CAR cell therapies, with several clinical trials recently initiated. However, the risk of immune-rejection caused by the mismatch of human leukocyte antigens (HLA) between the therapy and the patient poses a challenge to the continued growth of the clinical applications of iPSC-based cell therapies. Though solutions, such as HLA-banking or generation of iPSCs from each patient have been proposed, they are unrealistic given the costs and regulatory compliance required. Generation of immune-cloaked cells can be accomplished with gene editing technologies by preventing presentation of the HLA class I and II antigens on the cell surface, while at the same time overexpressing specific transmembrane proteins. This solution will greatly reduce time and expenses of cell therapies, with huge benefits for the patients. However, application of the CRISPR technology remains challenging for generating cells with multiple genetic alterations, especially in iPSCs which are sensitive to these procedures. Here we present a streamlined pipeline to produce gene edited iPSCs. We first determine the editing efficiencies of CRISPR complexes at target sites by performing deconvolution of Sanger traces. Once the CRISPR components are validated, iPSCs are single sorted and using imaging software for systematic monitoring we can assure single cell clonality. Clone screening for the correct edits is performed, followed by NGS analysis for validation. We have achieved up to 90% editing efficiencies using our optimized conditions and >30% recovery from single iPSCs. Our bespoke gene editing platform offers state-of-the-art facilities and fits in seamlessly with our good manufacturing practice (GMP)-compliant master cell banking and proprietary cell-specific differentiation pipelines.

P512

Studying TCR-pMHC interactions: Binding of full-length and computationally stabilized single-chain TCRs to a cancer epitope

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In the advent of targeted personal immunotherapy and T-cell based therapeutics interest in the T cell receptor (TCR) interaction with its natural ligand, the peptide loaded major histocompatibility complex (pMHC), has grown. As opposed to antibodies targeting extracellular antigens, TCRs can recognize intracellularly processed peptides and thus trigger T cell responses to malignant or infected cells. Despite their great importance in the natural immune response, the TCR-pMHC interaction remains not fully understood. Additionally, the heterodimeric nature of TCRs impedes handling as drugs and in research. This generates the need for single-chain TCRs (scTv) that combine the assets of their antibody counterparts, single-chain variable fragments (scFv), with the specific TCR binding properties. To address the low stability of scTvs, computational protein design was performed to increase construct stability and expression yield. Finally, to better study the interaction between the pMHC and its ligands, a binding assay was established and tested for TCRs and scTvs in comparison to epitope-specific antibodies for the known tumor epitope NY-ESO-$1_{157\text{-}165}$ bound to HLA-A0201. Single-chain TCRs were designed for improved stability with ProteinMPNN and Rosetta, starting from the known TCR 1G4 (PDB ID 2F53). The variable fragment was extracted, the whole sequence except the complementary determining region designed with ProteinMPNN and the most promising point mutations scored in Rosetta. Full-length TCR constructs were based on previously published stabilized versions of G4_122 (Froning, K., Maguire, J., Sereno, A. *et al.*, 2020). TCRs, scTvs and TCR-like antibodies (3M4E5 and 3M4F3) were expressed in a human embryonic kidney cell line. Biotinylated HLA-A0201 was expressed in *E. coli* and refolded with the NY-ESO-1 peptide SLLMWITQC. Binding was measured using biolayer interferometry (BLI). Computational protein design of the scTv led to improved expression rates for all chosen constructs compared to wild type. In addition, predicted improvement in stability correlated well with the final expression yields. A BLI assay was established and used to measure binding affinities between pMHC and full-length TCRs, scTvs and antibodies, respectively. Measured affinity of antibodies and TCRs agreed with the K_D values known from literature. In conclusion, we introduced a pipeline for the computational stabilization and expression of pMHC-binders, which can then be tested for their binding affinity in a robust BLI assay, thus facilitating the streamlined design and optimization of specific T-cell epitope binders for different pMHCs of interest.

P513

A simple and high reproducible approach for creating patient-derived xenograft models for developing novel treatment options in pediatric AML

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Childhood acute myeloid leukemia (AML) is a genetically and biologically heterogeneous disease that originates from the abnormal proliferation and differentiation of malignant myeloid progenitors. When untreated, AML results in rapid bone marrow failure and death. Despite significant progress achieved in understanding the pathogenesis of the different genetic causes of AML and the remarkable rise of overall survival rates to approximately 80% in the past decades, the probability of recurrent- or therapy-resistant (R/R) disease remains high, thus emphasizing substantial gaps in our knowledge regarding the pathogenesis of R/R AML and underlining the need for further research in representable preclinical models. Patient-derived xenograft (PDX) models allow performing functional studies on primary patient-derived AML cells. To develop such a model system, we intravenously injected NOD/SCID/IL2Rγ^{null} (NSG) mice strain with unsorted primary AML blasts, freshly obtained from the bone marrow or peripheral of 72 children and adolescence at the time of diagnosis (n=66) or relapse (n=6). No conditioning of the mice occurred before injection of the primary blasts. However, to avoid chronic xenogenic graft-versus-host-disease mediated by patient-derived T-cells in the graft, the BM or PB samples were incubated with a CD3 mouse-anti-human antibody. Mice were monitored for up to 180 days for engraftment of AML blasts and upon development of AML transplanted serially into secondary recipients. To confirm the validity of our PDX models in amplifying the original AML leukemic stem cells, the characteristics of the engrafted human AML blasts were compared after first and secondary transplantation to the initial patient blasts at diagnosis. To this end, flow cytometry, fragment-length analysis and Sanger- or Next-Generation-Sequencing (NGS) were used. In total, 72 patient material were transplanted in 299 mice (172 first- and 127 second or third transplantations). In our model, engraftment success was 55% for samples isolated at patients' initial diagnosis and 67% for relapsed material. The engrafted AML subtypes mostly originated from patients with French-American-British subtype M7 (87%) followed by M5 and M4 (each 67%). The probability of engraftment was higher in patients stratified as "higher risk" at initial diagnosis compared to patients defined as standard risk. Importantly, the percentage of engraftment did not correlate with the number of AML blasts injected. Comparative flow cytometry analysis of the initial and the engrafted blasts showed that engrafted AML blasts after serial transplantation resembled the phenotype of the initial samples but became more immature. In addition, except for one patient sample in three mice, all of the initial genetic markers including *WT1*, *GATA1*, *NPM1* or *CEBPa* mutations or *FLT3*-ITD as well as cytogenetic traits/translocations such as t(10;11) or inv(16) were unaltered in the engrafted AML blasts. No engraftment occurred for blasts carrying the translocations t(8;21) and t(15;17). Conclusively, our PDX platform significantly facilitates future research with primary AML blasts under settings closer to the reality, thus suitable to better understand the characteristics of AML stem cells and to test the efficacy of novel treatment options such as cellular immunotherapies.

P514

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 a CAR, which is able to specifically recognize and destroy tumor cells. However, current CAR T cell engineering is based on stable transduction with viral vectors. This can give rise to several concerns including high manufacturing costs as well as a permanent CAR expression, which can cause adverse immune reactions. Therefore, we aim to use lipid nanoparticles (LNPs) as a nonviral method to deliver mRNA molecules (encoding for CAR) into T cells and hereby induce transient CAR expression. Nevertheless, primary human T cells are known as hard-to-transfect cell types. Here, we investigated the influence of LNP charge and the cell culture medium composition on the transfection efficiency. Various LNPs, encapsulating eGFP encoding mRNA, were formulated including the ionizable C12-200 and cationic DOTAP lipids through microfluidic mixing. Furthermore, primary human T cells were isolated from buffy coats, stimulated, and kept in culture for up to 10 days. At set time points following activation, the cells were harvested and 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 adsorb 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 protein. Moreover, an increase in transfection rates was observed depending on the activation status of the cells. To further improve the yield, indicating the percentage of viable transfected cells, a range of mRNA doses was tested. Here, results showed an increase in transfection efficiency for higher doses of mRNA, while cytotoxicity was maintained low. On the other hand, the cationic LNPs transfected T cells independent of targeting proteins and activation status. Nevertheless, moderate cytotoxicity was observed, requiring further optimization of the cationic LNP composition. In conclusion, our results demonstrate that the combination of LNP charge, extracellularly available proteins, and T cell activation status has a profound impact on the transfectability of T cells by LNPs. Future research will be focused on evaluating mRNA-induced CAR expression and subsequent tumor cell killing efficiency.

P515

Towards engineered NK cell therapy: mRNA transfection of NK-92MI cells by photoporation

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Adoptive T-cell therapy is one of the fastest-growing branches of cancer immunotherapy. It is based on genetically modifying the patient's T cells for a more precise recognition and destruction

of cancer cells. This modification is done by introducing a chimeric antigen receptor (CAR). CARs are artificial protein receptors that can target any tumor-associated antigen. Currently, multiple CAR-T cell therapies are on the market for different hematological malignancies. However, as these therapies are still expensive and labor-intensive with severe side effects, there has been growing interest in the use of natural killer (NK) cells. As NK cells are part of the innate immune system, they offer a possibility for allogenic therapies with fewer side effects such as graft-versus-host disease and cytokine release syndrome. Despite viral transduction still being the most common method for the engineering of immune cells, non-viral transfection techniques are actively being investigated in order to avoid the high costs, safety hazards, and limited cargo capacity of viral vectors. Previously we have shown that nanoparticle-sensitized photoporation, an upcoming new non-viral physical transfection technique, is an effective method to deliver gene-modifying effector molecules in T cells. While non-degradable gold nanoparticles are commonly used as sensitizing nanoparticles for photoporation, we recently demonstrated that biodegradable polydopamine nanoparticles (PDNPs) can be used instead, paving the way towards the use of photoporation for the production of engineered therapeutic immune cells. On the other hand, there has also been an interest in limiting the exposure of the cells to nanoparticles, to avoid regulatory and safety concerns even more. For this reason, photothermal iron oxide nanoparticles can be embedded in biocompatible electrospun nanofibers (PEN), avoiding direct contact of the nanosensitizer and the cells to obtain a nanoparticle-free final cell product. While both techniques have been successful in the delivery of gene-modifying effector molecules in T cells, this has not been tested for NK cells. Therefore, in this project, we investigate the use of photoporation with PDNPs and PENs to deliver different macromolecules in NK-92MI cells with the promise of generating CAR-NK cell products.

PDNPs were synthesized and characterized (DLS and SEM imaging) as previously reported (Harizaj *et al.* 2021). Transfection efficiency of eGFP mRNA was evaluated as a function of PDNP concentration, reaching a maximal delivery yield of 29%. Thanks to being gentler to cells, this is 2-fold better than what we could achieve with Nucleofection.

Next, PENs were successfully synthesized and characterized as discussed before (Xiong *et al.* 2021). Preliminary results show that photoporation with the PEN-substrates allows for the delivery of large macromolecules such as eGFP-mRNA as well. Overall, our results confirmed that photoporation can be used to deliver macromolecules into NK-92MI cells with limited impact on cell viability, paving the way towards generating high-quality CAR NK cells.

P516

Engineering programmable, TME-responsive macrophages for solid tumour immunotherapy using Gene Editing induced Gene Silencing (GEiGS) technology

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In contrast to haemotologicial cancers, the efficacy of immunotherapy approaches for solid tumours has been limited, primarily due to antigen heterogeneity, reduced infiltration, and the immunosuppressive tumour microenvironment (TME). In solid tumours the presence of pro-tumoural and anti-inflammatory tumour associated macrophages (TAM) are generally associated with poor prognosis. TAMs are known to supress response to standard-of-care

therapeutics, including chemotherapy, immunotherapy and angiogenic inhibitor. The extent to which macrophage phenotype supports tumour growth is linked to the cues from TME.

Here we present a new 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 polarity in response to environmental cues.

GEiGS-engineered macrophages maintain the ability to home in on the tumour based on mechanisms underpinning TAM recruitment but use TME cues to turn on a pro-inflammatory programme once they reach solid tumour site, thus acting as a 'Trojan horse'. We present data on how GEiGS can be used to control the (pro- and anti-inflammatory) polarity of iPSC-derived macrophages. Furthermore, we utilise state of the art in-vitro models to demonstrate how gene silencing can be linked to extracellular cues associated 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. We believe this validates a core platform to develop next generation, adaptive cell therapies, addressing many challenges with current approaches.

P517

Harnessing the therapeutic potential of induced pluripotent stem cell-derived mesenchymal stromal cells: an immunomodulatory perspective

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Mesenchymal stromal cells (MSC) represent a stem cell population with promising therapeutic effects in a broad range of diseases involving tissue regeneration and the immune system. However, complexities related to donor variability, extensive in vitro expansion and different tissue sources continue to limit the use of MSC as an advanced medicinal product. To overcome these limitations, it is essential to establish methods that are more reliable and to identify accessible sources of MSCs. The generation of induced pluripotent stem cell (iPSC)-derived MSC (iMSC) appears to provide an opportunity to effectively address most of these limitations. In this context, we developed a cell-based assay platform to assess the immunomodulatory potential of iPSC-MSCs with respect to T cell proliferation and macrophage polarization. As a proof of concept, we conducted a comparative analysis with primary umbilical cord-derived MSCs and investigated relevant cell surface markers using flow cytometry. Strikingly, iPSC-MSCs exhibited a similar level of suppression of beads-stimulated peripheral blood mononuclear cells (PBMCs) proliferation in cell-cell contact or separated in transwells, compared to the in vitro reference population. Additionally, under inflammatory stimuli, iPSC-MSCs induced significant expression of the markers CD206 and CD163 in macrophages, confirming that the cells can promote the enrichment of M2-type regulatory macrophages. Finally, to investigate the underlying mechanisms, we assessed the expression of common MSC-derived soluble factors known to be involved in MSC mediated immune regulation in the cocultures using ELISA. Our data exhibits a decreased TNFα level and IL-10 induction, suggesting that iMSCs exert an immunomodulatory effect on T cells and monocytes to induce an anti-inflammatory response in which the secretome

(cytokines) could be playing a crucial role. The results suggest that iPSC-MSC holds considerable potential as an alternative source of MSCs in therapeutic applications because of their potent immunomodulatory properties.

P518

Pre-industrial production of a lentiviral vector for CD28/4-1BB CD123 CAR T cells

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Previously, a series of experiments in mice have provided a proof-of-concept that CD28/4-1BB CAR T cells directed against CD123 (CAR123 T cells) could efficiently and safely treat a model of blastic plasmacytoid dendritic cell neoplasm (BPDCN) (Bôle-Richard et al. Leukemia, 2020). In the perspective of clinical translation, the production of CAR123 T cells is now being developed to meet good manufacturing practice (GMP) requirements. Here, we describe the process for the production of the lentiviral vector (LV) (CAR123-LV). The cassette for this self-inactivated HIV-1-derived LV was fitted for clinical use. The CAR123-LV was pseudotyped with VSVG and produced in pre-industrial conditions at medium scale (8L) using transient transfection of adherent HEK293T cells in CF10 cell factory stacks. The production process was adapted from a previously published workflow (Merten et al. Human Gene Therapy, 2011). Plasmid DNA was removed by adding DNAse during the culture and before the harvest. The downstream purification and concentration process included clarification, anion exchange capture, TFF, diafiltration and sterile filtration steps. The process was reproducible in 4 separate 8L-scale runs. The infectious titer of the clarified harvest was 3.7±2 E+06 IG/mL and the process generated 12.6±4 mL of final product titrating 4.9±1.8 E+08 IG/mL, representing a 633 fold volumic concentration and an overall process yield of 24±11%. Total protein and plasmid DNA removal was demonstrated. The purified CAR123LV was stable and infectious for T cells (Jurkat T cells and primary T cells). The resulting CD123 CAR-T cells expanded and were functional, demonstrating specific cytotoxic activity in vitro. The feasibility of industrial manufacture of the CD123 LV supports further development of the CD123-specific CAR-T cells in GMP and towards clinical use.

P519

Efficient elimination of cells expressing allergy symptom-mediating IgE by treatment with specific CAR-NK-92 cells

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With permanently increasing prevalence, allergies have become a worldwide healthcare problem. Symptoms are very diverse and can range from relatively mild ones, e.g. itching and sneezing, via severe breathing difficulties and chronically impaired quality of life up to life-threatening allergic shock. In type I-allergic diseases such as allergic asthma, food allergies and urticaria, soluble allergen-specific immunoglobulin E is the mediator of the allergic symptoms. Immunoglobulin E is also present as part of the B cell receptor complex on immunoglobulin E-expressing B cells. In humans, one isoform of membrane-bound immunoglobulin E contains an additional 52-amino-acid stretch referred to as extracellular membrane-proximal domain (EMPD). We hypothesize that the supply of allergy symptom-triggering immunoglobulin E could be terminated by eliminating immunoglobulin E-expressing B cells using redirected immune-effector cells. To this end, we generated novel chimeric antigen receptors (CARs) with known EMPD-specific single chain variable fragments (scFvs) as antigen-binding domains. The novel CARs were cloned into retroviral vectors used to transduce human NK-92 cells. CAR-NK-92 cells were tested in luciferase-based cytotoxicity assays against an engineered human Burkitt's lymphoma cell line stably expressing membrane-bound immunoglobulin E with or without the EMPD. The generated EMPD-specific CAR-NK-92 cells were found to be highly functional *in vitro*, efficiently mediating antigen-specific lysis of target cells (up to 95 ± 1% killing within 4 hours at an effector to target cell ratio of 5:1). Following further *in vitro* and *in vivo* studies to characterize their safety and functionality, EMPD-specific CARs could be a promising option for the treatment of severe allergic diseases.

P520

Development of CAR-NK Cellular Platform for Cancer Immunotherapy

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Although Chimeric Antigen Receptor (CAR)-T cell therapy has proven to be successful in treating multiple haematological malignancies, their use is limited due to the potential life-threatening adverse events, including cytokine release syndrome (CRS), neurotoxicity and graft-versus-host disease (GVHD). CAR natural killer (CAR-NK) cells are rapidly emerging as a promising alternative cellular therapeutic with improved efficacy and reduced adverse effects. Moreover, due to the intrinsic characteristics of NK cells, whereby they lack antigen-specific cell surface receptors, CAR-NK cells offer the additional advantage of being an "off-the-shelf" product, thus satisfying the requirement for large-scale production for cancer immunotherapy, giving it the potential to reach more patients at a reduced cost.

Here we outline the method to generate, expand and enrich human primary anti-CD19 and anti-HER2 CAR-NK cells, and demonstrate their efficacy in-vitro against target-expressing cancer cells.

Human primary NK cells were isolated from healthy donors and transduced with either an anti-HER2 CAR or a clinically tested CD19-targeting CAR lentiviral vector (LV) or GFP expressing LV, as an empty vector transduced control. Transduction efficiency greater than 10% or 20% was achieved following transduction and expansion over a 7-day period in response to CAR LV or GFP LV respectively. Following sufficient expansion, cytotoxicity of the CAR-NK cells was tested in-vitro in a Tumour Killing Assay (TKA) against target-positive or target-negative cells. As expected, CAR-NK cells displayed increased cytotoxicity towards target-positive cells when compared to non-transduced or GFP transduced controls. This effect was not observed with

target-negative cancer cells, strongly suggesting CAR specific mediated killing. Moreover, CAR-NK cells showed increased degranulation and IFNγ secretion when compared to both non-transduced and GFP-transduced controls, suggesting increased NK cell activation and cytotoxicity. Additionally, when degranulation of CAR positive and CAR negative NK populations was directly compared, the level of NK cell degranulation was analogous between the two populations, suggesting that CAR NK cells can enhance the cytotoxic activity of neighbouring non-transduced cells, resulting in enhanced killing of target-positive cells by the total NK population present.

Taken together these data demonstrate the generation, expansion and enrichment of CAR-NK cells with enhanced cytotoxic activity towards target-positive cells. This culture system provides the scientific community a platform to screen novel CAR-constructs and to test novel immunomodulators which could enhance NK cell expansion or functional potential to further develop new, more widely available cellular therapeutics to treat cancer.

P521

Increasing the efficiency of lentiviral transduction to manufacture chimeric antigen receptor targeting HER2 in stirred-tank bioreactors

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Chimeric antigen receptors (CAR) are engineered fusion proteins that can be designed to be expressed in T cells targeting antigens expressed on cancer cells. 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. CAR-T cells have been used to treat patients with relapsed and/or refractory B cell lymphomas, B cell acute lymphoblastic leukaemia and multiple myeloma. However, while huge advances have been performed in the field of haematological malignancies, the efficacy of immunotherapies targeting solid tumours remain challenging due to the harsh microenvironment of the tumour and the lack of defined target antigens. 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 more efficient CAR-T cell manufacturing processes, particularly focusing on increasing the efficiency of the LV transduction step, could contribute to increase the number of patients potentially benefiting from these cell-based therapies. Thus, it is critical to optimize LV production, decreasing the inherent costs associated to their manufacture.

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 have 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, being also used for prognostic and as a predictive biomarker.

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). Additionally, several LV productions were performed combining a regular gag-pro-pol expression gene with a mutated one at the frameshift region to evaluate its impact in the ratio of transducing units/physical particles of the LV preparations and its possible quality improvement.

To increase the efficiency of LV transduction, the impact of distinct timing of LV transduction following T cell activation are also investigated. Additionally, selection of adequate cell culture media and agents that could act as transduction enhancers have the potential to increase the efficiency of the T cell transduction step performed in stirred-tank bioreactors.

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.

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P522

Novel module designs to promote CAR Regulatory T cell persistence

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$CD4^+$ $CD25^+$ $CD127^{low/-}$ $FoxP3^+$ Regulatory T cells (Tregs) are important suppressor cells of the immune system. Tregs are a subpopulation of T cells which maintain immune homeostasis by inhibiting T cell proliferation and controlling their cytokine production, mainly by inhibiting pro-inflammatory cytokine production. Clinical trials have employed expanded polyclonal Tregs as a means of treatment for patients with transplant rejection. Data from various studies have shown that allospecifc Tregs offer greater protection from graft rejection compared to polyclonal Tregs. Chimeric antigen receptors (CARs) are synthetic fusion receptors that can be used to redirect the specificity of Tregs to designated antigens. CAR Tregs have been designed with the aim for treating patients with transplant rejection as well as various autoimmune diseases. A key success factor of CAR Tregs immune suppressive response is how long the CAR Tregs exist after infusion into the recipient to continue to exert their immunoregulatory effects over time. Therefore, despite a well-functioning CAR Treg, poor persistence of CAR Tregs can limit the effectiveness of the cell therapy. Thus, an area of important research pertains to increasing the persistence of CAR Tregs once injected back into the recipient. IL-2 cytokine plays a crucial role in Treg generation, survival, maintenance, expansion and stability. IL-2 induces high phosphorylated STAT5 (pSTAT5) levels within Tregs which signals to promote pro-survival protein production amongst other cellular events. We have designed and engineered novel modules which can provide Tregs with enhanced persistence by exploiting the pSTAT5 signalling pathway. Our persistence technologies provide a constitutive signal; these designs encompass different signalling pathways. Furthermore, we have different molecular approaches which include a tethered cytokine and a receptor

dimerization tool which can be utilised in different settings depending on the strength of pSTAT5 signalling required. Our data show that transducing Tregs with these persistence technologies clearly extended persistence of transduced Tregs over control Tregs without the persistence technologies. Our results show that these CAR Tregs with the persistence technology maintained their suppressive function and phenotype. Furthermore, we have validated these persistence technologies in humanised *in vivo* models which showed enhanced persistence of Tregs transduced with the persistence technologies compared to control Tregs. Collectively, our data show that our persistence technologies can be used in Tregs as a means to promote their survival and persistence whilst maintaining their suppressive function and stable phenotype.

P523

Optimisation of culture conditions and limit of detection-determination of IL-2 dependency assay

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The use of chimeric antigen receptor (CAR) T-cell therapies has shown great potential for the treatment of haematological malignancies with high target specificity. As such, the number of therapeutic CAR T-cell products entering clinical trials in both oncology and now non-oncology settings is increasing. Commonly used method for engineering CAR T-cells including genome editing (e.g., CRISPR-Cas 9), and lentiviral transduction, are associated with safety risks including off target editing and insertional mutagenesis increasing the potential for T-cell clonal expansion and malignant transformation. Preclinical assessment of these products is inherently challenging and the need for improved assays is crucial to identify mutagenic safety related events that may occur in your products before they enter human trials. T-cells that undergo malignant transformation have been shown to transition from IL-2 dependent to IL-2 independent growth, laying the ground for the IL-2 dependency assay to assess potential transformation. By evaluating different cell culture conditions in the presence and absence of IL-2, we present an optimized IL-2 dependency assay for the detection of transformed cells in our CAR T-cell products. In addition, we evaluated the sensitivity of our assay through a series of spike in experiments whereby transformed cells were seeded into the assay at various ratios and clonal outgrowth monitored. This sensitive *in vitro* assay could be a useful tool for predicting malignant transformation particularly as we moved towards more extensively engineered CAR T-cell therapies products in the future and reduce the need for more complex models including *in vivo* assays.

P525

Validation of the sterility test using BD BACTEC FX40 in frozen CAR-T cells product and comparability to the compendial direct inoculation method

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The purpose of this study was to validate the BD BACTEC FX40 device for sterility testing of the CAR-T drug product (frozen product containing freezing media) and to demonstrate that the method is comparable to the compendial sterility method (Ph.Eur. Chapter 2.6.1 and USP <71>; direct inoculation to the medium).

In the first part, culture conditions (addition of FOS supplement) and technique of vials inoculation to the BACTEC vials was optimized, especially for anaerobic strains (*Clostridium sporogenes*).

Subsequently, the comparability of the two methods was demonstrated with three batches of the product and microbial concentration 100 CFU of nine microorganisms (*Clostridium sporogenes*, *Cutibacterium acnes*, *Staphylococcus aureus*, *Streptococcus pyogenes*, *Brachybacterium nesterenkovii*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans*, *Aspergillus brasiliensis)*.

All cultivation vials containing microorganisms were tested positive in the BACTEC™ device. Time of positivity was within 4 days for all microbial strains. Results from BACTEC testing of the drug product were comparable to the compendial sterility test.

Finally, the comparability was assessed at lower CFU levels, down to 10 CFU in an additional batch of the product.

Sensitivity and specificity were evaluated for the BACTEC method. For sensitivity, the two methods were compared for detection of bacterial and fungal contaminants at two concentrations - 10 CFU and 50 CFU/vial. Specificity was verified with samples spiked with 50 CFU of microorganisms (positive) and unspiked samples (negative).

This study confirmed that the BACTEC method is able to detect bacteria or fungi inoculated at 10 CFU while in some cases the compendial sterility test did not lead to a positive result at this concentration. Time to positivity was within 4 days for all microbial strains. Results from BACTEC testing of the drug product were comparable to or better than the compendial sterility test. Negative samples were detected as negative confirming the specificity of the method.

In addition, it was confirmed that all microorganisms which were detected by the BACTEC device were also able to grow in the subsequent subculture and were detected on blood agar and in culture medium in the bottles.

In conclusion, The BACTEC sterility fulfilled all criteria for comparability and validation as requested by the Pharmacopoiea.

However, on the top of this scope the testing of other CR specific environmental bacterial or fungal species is encouraged.

The most conservative approach is to combine the BACTEC testing with the compendial method and release conditionally the product based on the fast testing by BACTEC (after 5 days) together with compendial sterility method (14 days) for the final release.

P526

Supporting CAR-T cell therapy development from discovery to IND-filing

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Engineered T cell therapies such as Chimeric Antigen Receptor (CAR) T cells and T cell receptor (TCR)-engineered T cells have emerged as a promising cancer therapy. To date, six CAR-T cell products have been approved by the FDA for the treatment of hematological malignancies. Many more T cell therapy products are currently being explored, directed towards both liquid and solid tumors as well as for other clinical indications.

Due to its innovative nature and the complex biology involved, CAR-T cell therapy product development can face many challenges that may delay the discovery programs, starting from the identification and characterization of the binder to use and the selection of a lead candidate. While most CAR programs rely on precursor molecules (scFV) affinity screening, this has proven to not always correlate best to the derived CAR-T activity observed in vivo afterwards. However, screening a large number of CAR constructs directly in T cells seems impracticable due to the lengthy timelines for their production and the high costs associated with generating viral vectors. To overcome current limitations and streamline the lead selection, we offer alternative screening methodologies that allow to specifically identify, design, and optimize binders for the target of interest as well as testing their performance in vitro. Binding avidity can then be assessed using the z-Movi technology to aid optimal lead candidate selection as avidity has proven to be a better predictor of in vivo efficacy and persistence.

Furthermore, the lack of pharmacologically relevant animal models emphasizes the need to evaluate the safety of CAR-T cell therapy products in vitro. An important step is the identification of off-targets using the Retrogenix cell microarray technology that can be used to select leads with highest specificity or to characterize the selected lead. In line with the regulatory agencies' guidelines, in vitro co-culture assays were developed using a variety of primary or iPSC-derived human cells (representing various tissues including vital organs) to assess potential off-target and on-target/off-tumor effects of the cell therapies against healthy cells. Lastly, the cells can be tested in vivo to assess their efficacy in a more representative system and gain insights in their bio-distribution, homing sites, prevalence, and differentiation.

Additionally, the production, characterization, specificity, and efficacy testing of T cell therapies remain of central importance for the product development. For that purpose, Charles River Laboratories combines early-stage R&D scientists, safety advisors and CMC experts in GMP manufacturing and release testing, resulting in better control during the whole process.

In conclusion, Charles River Laboratories provides an end-to-end optimized pipeline to aid early-stage lead discovery, optimization, and development of CAR-T cell therapies to support Investigational New Drug (IND) applications in accordance with the regulatory requirements.

P527

Building a robust and efficacious immune cell therapy pipeline beyond oncology

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AstraZeneca Biopharmaceuticals R&D Cell Therapy department develops cell-based therapies to address cardiovascular-, renal-, metabolic-, respiratory-, immunological- and rare diseases. Our main aims consist of regenerating and repairing diseased tissue, modulating the immune system or selectively eliminating dysfunctional cells. To achieve these goals, we are working with a variety of cellular platforms and equipping them with emerging technologies such as gene editing and synthetic biology. To leverage the latest scientific discoveries, we have established collaborations with key research institutes and innovative biotech companies.

One of our major ambitions is to modulate the immune system to restore balance in autoimmune and inflammatory diseases. In this context, we are focusing on T regulatory (Treg) cells, a population of lymphocytes which regulate and dampen the function of other immune cells. In immune-mediated diseases, Treg cells are outnumbered or unstable, which shifts the balance towards auto-reactive immune cells that attack healthy tissues. Our goal is to stabilize and expand Treg cells *ex-vivo*, so that when they can effectively rebalance the immune system once infused into patients, and therefore address the high unmet need in patients with immune-mediated diseases.

P528

Novel isolation and activation platform with active-release technology for scalable cell therapy manufacturing

Gibco™CTS™Dynabeads™CD3/CD28 has been used successfully in advancing T cell therapies through >200 clinical trials and is also utilized in commercial T cell drug manufacturing. Our next generation Gibco™ CTS™ Detachable Dynabeads™ CD3/CD28 combines the excellent performance of CTS Detachable Dynabeads CD3/CD28 with the flexibility of an active release mechanism, enabling termination of activation signaling by releasing the Dynabeads at any time point using the CTS Detachable Dynabeads Release Buffer

The bead release technology is critical to i) control activation time and preserve a young phenotype, ii) release Dynabeads prior to T cell manufacturing steps where the presence of beads is unwanted, and iii) to enable shortening of T cell manufacturing process to a few days. CTS Detachable Dynabeads is compatible with the closed and automated Gibco™ CTS™ DynaCellect™ Magnetic Separation System.

P529

Optimized Poxviral promoters improve anti-tumor activity of Modified vaccinia virus Ankara expressing IL12 in mice

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Synthetic or natural poxviral promoters are required to drive transgene expression by recombinant Modified vaccinia virus Ankara (MVA) vectors, that take place in cytoplasmic viral factories. We designed several optimized promoters, carrying different combinations of early and late elements, that were tested for their ability to drive strong transgene expression in the context of MVA-based vectors both in vitro and in vivo in murine tumor models. Here we show that the strength of the promoter chosen to drive expression of the cytokine IL12 impact the antitumor activity of MVA-IL12 vectors, with sustained local cytokine production needed to obtain significant therapeutic efficacy. SynE1 promoter, containing 4 tandem optimized early elements fused to one strong late synthetic element, was selected since it showed very high IL12 expression levels in MVA-infected human and murine tumor cell lines and human THP1 monocytes/macrophages. Notably, SynE1 allowed relevantly higher and more persistent in vivo transgene expression compared to the well-known early/late P7.5 poxviral promoter. Intra-tumoral delivery of MVA encoding IL12 driven by SynE1 (MVA-SynE1-IL12) but not MVA-P7.5-IL12 led to complete remission of established tumors in multiple murine models, including those resistant to the activity of checkpoint inhibitors (CPI) even when used at a low dose translatable to humans. This is accomplished thank to the immunomodulatory proprieties of both the MVA vector and the sustained levels of encoded IL12, that co-operate to switch the tumor microenvironment from immunosuppressive into immunostimulatory.

P530

Zeb1 downregulation sensitizes pancreatic cancer-associated fibroblasts to killing by oncolytic reovirus through upregulation of the reovirus receptor Junction Adhesion Molecule A

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Pancreatic tumors display an abundance of cancer-associated fibroblasts (CAFs), which negatively affect prognosis and therapy response. Oncolytic virotherapy exploits viruses that preferentially lyse epithelial cancer cells as opposed to normal cells. Interestingly, we have observed that oncolytic reoviruses are able to infect and lyse CAFs, in addition to epithelial cancer cells. Targeting CAFs, in addition to cancer cells, could be advantageous to increase therapy effectiveness. It could serve as a conduit for viral spread and simultaneously disrupt the desmoplastic barrier around tumors, thereby also accelerating the influx of other therapeutics and immune cells. We previously found that the proneness of CAFs to lysis by oncolytic reovirus

correlates with the cell surface expression levels of the reovirus entry receptor junction adhesion molecule A (JAM-A). However, most pancreatic CAFs do not express JAM-A.

Therefore, a genome-wide CRISPR/Cas9 screen was employed to identify the genes regulating JAM-A expression on fibroblasts, which can subsequently be targeted to sensitize CAFs to reovirus. Pancreatic stellate cells with a moderate JAM-A expression level were transduced with a gRNA library making a knockout of one gene per cell. The highest and lowest JAM-A expressing cells were sorted and sequenced to identify the gRNAs that regulate JAM-A expression. Clonal CRISPR/Cas9-generated knockouts of a top negative regulator were generated and infected with reovirus, followed by cell viability assays to quantify their susceptibilities to reovirus-induced cell death.

F11R, the gene encoding JAM-A, was identified as the top positive regulator of JAM-A expression in the CRISPR/Cas9 screen, verifying the validity of the screen. The top negative regulators identified were Fibroblast Growth Factor Receptor 1 (FGFR1) and Zinc finger E-box Binding homeobox 1 (Zeb1), thereby serving as potential therapeutic targets to sensitize CAFs to reovirus treatment. Using clonal Zeb1 knock-outs, Zeb1 was confirmed as a strong regulator of JAM-A expression. Zeb1 knockout in JAM-A negative pancreatic fibroblasts caused a robust upregulation of JAM-A and sensitized these inherently resistant fibroblasts to reovirus-directed cytolysis. Additionally, the clinically approved drug Mocetinostat, previously described to inhibit Zeb1, upregulated JAM-A expression on CAFs and increased cell lysis by reovirus.

Altogether, our data show that Zeb1 is a strong negative regulator of JAM-A expression on fibroblasts and that Zeb1 inhibition can sensitize CAFs to reovirus-induced cell death. This research provides a rationale for combining Zeb1 inhibitory drugs with oncolytic reovirus treatment to improve killing of CAFs, which in turn could boost overall tumor eradication.

P531

Phage-guided oncolytic virotherapy and cancer vaccines

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Therapeutic cancer vaccines have shown tremendous potential in the treatment of various cancer types. These vaccines work by deploying a range of diverse immunotherapeutic strategies that can stimulate an immune response to selectively eliminate cancer cells this includes tumour antigens. Tumour antigens for many years were used as target for therapeutic cancer vaccines due to their ability to efficiently activate the immune system. However, tumour antigens are often difficult to elicit an effective immune response, they are not found in all types of cancers and their expression can be lost as tumours grow. Highly immunogenic and more tumour specific antigens and vaccine development platforms need to be established. Viral antigens have emerged as a promising alternative to tumour antigens for the development of therapeutic cancer vaccines due to their ability to efficiently elicit an effector as well as memory T cell response specific to these antigens. Moreover, efficient delivery of antigens requires suitable vaccine platforms as this will result in an effective therapeutic cancer vaccine. Nucleic acid vaccines have shown great success in infectious diseases and are being tested in cancer. However, successful nucleic acid vaccination against cancer will depend on successful development of efficient and selective nucleic acid delivery vectors. A Transmorphic Phage/AAV (TPA) particles, based on a hybrid between the M13 phage capsid and inverted terminal repeats (ITRs) elements of human adeno-associated virus (AAV2) virus has been successfully developed by our Phage Virotherapy group. The coat proteins of M13 were designed to display the RGD4C ligand that directs the phage particles to target

tumour cells and tumour-associated vasculature through the binding to $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin receptors. These receptors are overexpressed on tumour cells and tumour-associated vasculature. Here, using this novel transmorphic particle system we cloned a fusion DNA sequence of two human viral antigens in the AAV2-based transgene cassette to target tumour specifically and induce significant immune response. Firstly, we explored the therapeutic efficacy of the systemic administration of RGD4C.TPA expressing the viral antigens in tumour-bearing C57B1/6 mice. Then, we investigated the TPA expressing the viral antigens can induce -specific immune responses against antigen 1 and antigen 2 in tumour-free mice. Our results demonstrate selective and efficient gene delivery of the viral antigens to the tumour cells, without harming healthy organs. Also, our strategy was able to show significant humoral and cellular immune response as well as induce sufficient therapeutic effect and inhibit tumour growth. Our transmorphic particle system provides a promising modality for safe and effective delivery of viral antigens and elicit a stable immunogenic response over time against these antigens in tumour mass.

P532

Development and testing of lipid nanoparticles as in vivo vehicle for solid tumor gene therapy to improve subsequent CAR T cell therapy

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Despite advances in the field, an increasing burden of disease from solid tumors is being reported worldwide. Limited treatment options are available in many high-risk cases and relapse situations. Adoptive T-cell therapy, utilizing chimeric antigen receptor (CAR) T cells, holds promise as a potential approach to treating solid tumors. However, significant obstacles need to be overcome, including inadequate T-cell infiltration and an immunosuppressive tumor microenvironment. To address this challenge, our research group is developing a personalized gene therapy approach that involves modifying a patient's tumor cells to express transgenes encoding immune modulators. These modifications aim to alter the tumor microenvironment, enabling CAR-T cells to better reach the tumor cells and, with enhanced activation, effectively destroy them. The objective of this subproject is to develop a targeted lipid nanoparticle-based delivery system (tLNP) for in vivo application of CRISPR-based gene therapy. The delivery system aims to package and transport both Cas9 mRNA, sgRNA, and single-stranded donor DNA templates at the same time to the tumor cells, facilitating tumor-specific CRISPR-mediated homology-directed-repair dependent knock-in. To date, we have successfully demonstrated the co-transfer of the gene therapeutic agents (Cas9 mRNA, sgRNA, and donor DNA template) using cationic lipids in vitro and are currently evaluating different LNP formulations to improve packaging efficiency. For in vivo application, the most promising candidates will be modified with tumor-specific antibodies to preferentially deliver the gene therapy to the tumor cells. Overall, our synergistic approach, combining immunotherapy with gene therapy, overcomes the obstacle of reaching all tumor cells, which has been a significant challenge in gene therapies for solid tumor treatment.

P534

Living anticancer Clostridium sporogenes colonizes in vitro 3D necrotic spheroids

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Most solid cancers contain areas of necrosis resulting from chronic hypoxic injury. These high-risk tumour characteristics are associated with poor prognosis due to increased risk of metastasis and resistance to chemotherapy and radiotherapy. Strikingly, this necrotic environment also provides an opportunity for targeted therapy, such as our alternative anti-cancer approach that uses the harmless anaerobic bacteria *Clostridium sporogenes (C. sporogenes)*. Upon injection as spores, this species selectively penetrates necrotic areas and germinates into metabolically active vegetative bacteria that can produce anticancer effects inside the tumour. We have developed innovative genetic tools to further enhance these effects by "arming" *C. sporogenes* with therapeutic genes such as immune checkpoint inhibitors and cytokines. Novel therapeutic strains can be generated effectively in a short period of time. Therefore, it would be essential to evaluate and validate them in robust systems prior to *in vivo* studies because considering the Three Rs principle of animal research, the use of animals should be reduced or replaced if possible. It would thereby be beneficial to develop an *in vitro* validation method that can mimic tumour necrosis as an intermediate step. To do investigate this, the Lewis Lung Carcinoma (LLC) cell line was used to generate 3D spheroids. Spheroids were first investigated on the development of necrotic fractions. Next, 5,000 colony-forming unit per millilitre (CFU/mL) of spores were added on different days to determine and optimize the experimental time frame of our spheroid model. The CFU/spheroid was determined for each time point. Microscopic images were taken every day to follow-up spheroid growth. Furthermore, a *C. sporogenes* strain expressing the fluorescent reporter gene unaG was investigated as a live imaging tool in the spheroids. Our results showed that LLC spheroids develop a necrotic core around day 5 post seeding. When spores were added on this day, *C. sporogenes* colonized the spheroids up to 72 hours, with 48 and 72 hours showing the highest number of bacteria inside the spheroids. Colonized spheroids started to disintegrate around 96 hours as a result of *C. sporogenes* colonization. Additionally to these results, the fluorescent unaG strain was live imaged in the spheroid model. Fluorescent intensity positively correlated to CFU/spheroid and fluorescent signal was diminished after spheroid disintegration. These promising results show the high potential of 3D *in vitro* models that can potentially serve as important intermediate validation step before embarking *in vivo* studies. These models will be applied for testing therapeutic efficacy and antibiotic sensitivity of our *C. sporogenes* strains.

P535

Targeted suicide gene therapy for cancer

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Cancer is a group of diseases where cells undergo relentless and uncontrolled division. Targeted cancer therapy refers to a type of treatment that focuses on specific molecules or pathways involved in the growth and spread of cancer cells. As cancer progresses, it typically undergoes increased heterogeneity that can be explained, among other reasons, by the concept of Cancer Stem Cells (CSCs). CSCs exert a pivotal influence on tumor heterogeneity, given their crucial role in tumor maintenance, proliferation, self-renewal, chemotherapy resistance, and metastasis. These features make CSCs an attractive molecular target which could improve current treatments by minimizing metastases and recurrences. Unlike traditional chemotherapy, which affects both healthy and cancerous cells, targeted therapy aims to selectively disrupt the cancer cells' ability to grow and survive while minimizing damage to normal cells. In this particular setting, gene therapy can be used to directly target CSCs, enhance the immune system's ability to fight cancer, and deliver therapeutic molecules to the tumor site.

Currently, one of the most interesting therapies strategies is direct suicide gene therapy. It consists in introducing a gene responsible for producing a toxin that can internally destroy the cancerous cell. Moreover, promoters, regulatory regions that direct the transcription of messenger RNA, are essential for directing the expression of therapeutic genes toward target tissues. They are therefore a feature that determines the success and efficacy of gene therapy in cancer. Survivin promoter is highly expressed in most cancers and even more in CSCs, while it is rarely expressed in normal adult tissues. All these characteristics make it an interesting candidate to control *ldrB* suicide gene expression. In the present work, our purpose was, on the one hand, to construct a cancer-specific vector that integrates the *ldrB* gene sequence under the control of survivin promoter and, on the other hand, to analyse the bio-efficiency of Survivin as a cancer-specific molecular target against tumor cells and CSCs versus non-tumor cells. For this, *in vitro* assays were performed in 2D and 3D culture models and *in vivo* assays using NOD SCID mice. Tet-On 3G systems for Doxycycline-inducible *ldrB* gene expression was used as control. Our results showed that *ldrB* gene expression under the control of survivin promoter causes a significant inhibition of HeLa and MCF-7 CSCs proliferation *in vitro* in both 2D and 3D models. Furthermore, these data are consistent with the observed decrease in cell viability using the ATPlite assay. Moreover, *in vivo* studies under the control of the survivin promoter induced a severe loss of proliferation in vivo without side effects in our animal model. Given our findings, the combination of the *ldrB* gene and Survivin promoter holds promising potential as a viable option for future therapies in breast and cervical cancer.

P536

Therapeutic efficacy of prodrug activator gene therapy using retroviral replicating vectors in experimental human ovarian cancer

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Introduction
Retroviral replicating vectors (RRVs) have been shown to achieve tumor-selective replication, efficient tumor transduction, and enhanced therapeutic benefits in a wide variety of cancer models. Early-phase clinical trials of RRV-mediated prodrug activator gene therapy using yeast cytosine deaminase (RRV-yCD) and 5-fluorocytosine (5-FC) prodrug demonstrated encouraging evidence of therapeutic benefit in recurrent high-grade glioma, and a subsequent Phase 3 trial demonstrated significantly increased survival in particular patient subgroups. Here, we tested two distinct RRVs derived from the gibbon ape leukemia virus (GALV) and the amphotropic murine leukemia virus (AMLV), which use different cellular receptors (PiT-2 and PiT-1, respectively) for viral entry, in human ovarian cancer cells.

Materials & methods
Expression levels of the cellular receptors for AMLV (PiT-2) and GALV (PiT-1) in ovarian cancer cell lines (A2780, Caov3, RMG-1, SKOV-3), HEK293 cells, and fibroblast were evaluated by quantitative RT-PCR. In vitro replication of RRVs expressing the green fluorescence protein gene (RRV-GFP) was monitored by flow cytometry and 5-FC-induced cytotoxicity was quantitated by AlamarBlue assay in RRV-yCD-transduced ovarian cancer cells. In vivo antitumor effect of RRV-mediated prodrug activator gene therapy was investigated in a SKOV-3 subcutaneous tumor xenograft model in nude mice.

Results
Quantitative RT-PCR analysis revealed substantial expression levels of PiT-2 and PiT-1 receptors in the RMG-1 and SKOV3 ovarian cancer cell lines, as compared to normal and non-malignant cells. Both RRVs showed efficient viral replication and spread in RMG-1 and SKOV3 cells, resulting in over 90% transduction by Days 10–13. Additionally, both RRVs showed efficient cell killing of RMG-1 and SKOV-3 cells in a dose-dependent manner when treated with the prodrug 5-FC. Notably, RRV-mediated prodrug activator gene therapy achieved significant inhibition of subcutaneous SKOV-3 tumor growth.

Conclusion
These data indicate the potential utility of RRV vector-mediated prodrug activator gene therapy in the treatment of human ovarian cancer.

P537

Novel replication-competent adenovirus 11p vector expressing adenovirus death protein at the E1 region triggers crucial tumor suppressor pathways in the metastatic prostate cells

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Novel replication-competent adenovirus 11p vector expressing adenovirus death protein (RCAd11pADP) at the E1 region manifested a strong tumor-killing effect on prostate cancer cells in both vitro and in vivo animal models. In the present study, we further investigated the molecular mechanisms from the whole transcriptome, focusing on genes involved in cancer cell pathways. The cellular gene expression in metastatic prostate cells infected by RCAd11pADP, RCAd11pRFP, and Ad11pwt was studied using a sensitive Ampliseq total RNA sequencing method. About 4000 differentially expressed genes (*DEGs*) with more than 2-fold changes were identified, and the expression profiles of those DEGs with potential anti-tumor effects in cells at 8, 24, and 72 hr p.i. were compared. Significant under-expressed genes found in prostate cancer were observed, and they are involved in the TNFα signalling pathway via TN-κB, Kras signalling pathway, epithelial-mesenchymal transition (EMT), androgen response, and transforming growth factor beta receptor signalling pathway. De-regulated genes in the p53 signalling pathway manifested dominant function in the virotherapy. Nineteen genes that play a critical role in virotherapy for prostate cancer were further validated using an RT2 profiler PCR array. We observed RCAd11pADP vector-mediated tumor suppression mainly via over-expressed five genes and under-expressed 14 genes. Meanwhile, therapeutic tumor suppression of other oncogenes was also revealed after infection of RCAd11pADP. Notably, the ADP insertion site in the E1 region of the vector showed more substantial tumor suppression than in the E3 region. Consequently, our findings suggest that RCAd11pADP is a potent vector candidate for use as an antitumor agent for clinical trials.

P538

Development of a gene therapy cell death-inducing system regulated by microRNAs for cancer

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Cancer is the second cause of premature death globally, and recent trends indicate it may become the leading cause of death in most countries over the course of this century.

We aim at developing a gene therapy platform (miRTo) for the development of personalized medicine, to treat cancer patients effectively and safely.

The miRTo technology consists in the specific expression of a cell death-inducing gene into cancer cells and their selective killing. The targeting specificity of this technology stems from the specific expression of endogenous microRNAs (miR) in cancer cells that are deployed on miRTo to regulate the expression of the cell death-inducing gene specifically in these cells.

We aim at developing the miRTo technology and validating miRTo therapeutic potential in T-cell acute lymphoblastic leukemia (T-ALL), an aggressive high-risk sub-type of ALL with high unmet clinical need, through the development of miRToTALL. Notably, our technology is designed in a manner that allows flexibility through easy-to-introduce modifications, enabling us to expand miRTo to other cancer types.

miRTo design consists of a bidirectional lentiviral (LV) vector, in which the killing gene is expressed by one promoter (EF1α), and an inhibitor of the killing gene promoter (PGK) is expressed by the second promoter, resulting in the constitutive repression of the system. For the development of miRToTALL, the expression of both mRNAs will be regulated by T-ALL-specific miRs. We built a prototype of miRTo using as backbone a bidirectional LV vector and used the Lac operon system as the repressor. We cloned Lac operator sequences (LacO) downstream of the EF1α promoter and cloned the Lac repressor (LacI) downstream of the PGK promoter. For proof-of-concept purposes, we used a reporter gene, GFP, as a surrogate marker for the killing gene. Using a provisional T-ALL-specific miR expression profile (obtained by comparing miR expression in T-ALL cells versus a limited number of normal cells), we have built trial versions of miRToTALL, using different combinations of miR target sequences (TS). We cloned in tandem 4 TS of miRs specifically downregulated in T-ALL cells (miR-29a and miR-149) downstream of GFP, and cloned 4 TS of miRs specifically upregulated in T-ALL cells downstream of LacI (miR128a and miR153).

We evaluated efficiency and specificity of the different miRToTALLs to selectively target leukemia cells *in vitro*. We performed cultures of T-ALL and non-T-ALL cells transduced with miRToTALL and analyzed GFP expression by flow cytometry. In target T-ALL cells, miRToTALL prototypes with TS for miR-128 and/or miR-153, and in particular those without TS for miR-29a, induce GFP expression. In contrast, in non-target cells, miRToTALL prototypes with TS for miR-29a and/or miR-149, and in particular those without target sites for miR-128 did not induce GFP expression.

Our results validate the feasibility and effectiveness of miRTo technology. We have now established T-ALL-specific miR signatures by bioinformatics analysis of publicly-available datasets obtained from T-ALL and normal cells (comprising 615 samples and 2620 miRs). These signatures have being quantitatively validated by ddPCR and will now be used to build the final miRToTALLs to be tested both *in vitro* and *in vivo*.

P539

A capsid-engineered conditionally replication-competent oncolytic adenovirus to antagonise cancer cell's immune checkpoint CD47

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CD47 is an anti-phagocytotic immune checkpoint marker on healthy tissue. Upregulation of this molecule is exploited by a multitude of haematological and solid cancers to evade immune surveillance and has also been implicated in tumour dissemination and metastasis. The promising antibody-mediated blockade is currently being evaluated in numerous clinical trials. However, the ubiquitous expression of CD47 not only leads to severe side effects such as anaemia and thrombocytopenia in systemic application, but also to an antigen sink that weakens efficacy and requires frequent administration.

We aimed to overcome these limitations by a capsid-engineered conditionally replication-competent oncolytic adenovirus based on type 5 (OAd).

A Notch-sensitive uPAR-regulated E1A promoter construct (NuPAR) was used to achieve tumour specific replication. The latter was indicated in first experiments with healthy primary nasal epithelial cells.

The virus was designed to encode a secreted CD47 high affinity ligand (SIRPα-Fc) in a replication-dependent manner controlled by the major late promoter (MLP). Comparative evaluation of the transgene expression kinetics unveiled that the MLP-driven expression cassettes were exclusively active in replication-competent OAds but silent in replication-deficient counterparts (Ads).

Secretion of high levels of the recombinant protein was corroborated in Western blots and affinity chromatography of the supernatant. Flow cytometric analysis revealed a specific blockade of CD47 in up to 99 % of the infected and bystander $CD47^+$ cancer cell lines A549, MDA-MB231, and KM12 at 1 to 15 multiplicities of infection (MOI). Incubation of uninfected A549 with transferred supernatant from infected A549 (at 3 MOI, 48 h) indicated a potent bystander effect with SIRPα-Fc product detected on > 70 % of the uninfected cells. Importantly, non-specific targeting was successfully circumvented as proven in the $CD47^-$ HepG2 and CACO2 cell lines.

The oncolytic potency of the different virus variants was assessed by crystal violet assays and confirmed using A549 and MDA-MB231 cell lines.

Moreover, tumour cell killing by the CD16^{-}SIRPα^{+} NK92 cell line was significantly enhanced upon infection of the malignant cells ($p < 0.004$) as determined by flow cytometry and spectrometry. Ongoing killing- and phagocytosis assays with primary blood mononuclear cells exhibited nearly doubled natural killer cell cytotoxicity against cocultured tumour cell lines when exposed to the SIRPα-Fc in vitro.

Finally, the vectors were packaged into capsids optimised by a combination of genetic and chemical shielding and targeting technologies. This ensures maximum efficacy for intravenous application. Of note, the multitude of mutations in the vector genome was fully compatible with vector production.

P540

Harnessing the power of multiomics from a single sample with advanced automation for sample handling and processing

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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 window into the immune system that, when combined with these omics tools, can provide a near holistic view of immune processes across patient cohorts.

Here we detail a workflow using a single blood draw to rapidly produce a diverse set of multiomics results including genomics, epigenomics, transcriptomics and proteomics. This starts with automated sample handling and processing of the primary blood draw to ensure high viability and yield of PBMCs, along with simultaneous plasma separation and collection. These samples are then aliquoted and simultaneously processed for automated and semi-automated whole exome sequencing, single cell RNA sequencing, epigenetic characterization and Olink biomarker assays. With this robust workflow and advanced robotics for sample handling and processing that minimize potential batch effects, all these datatypes can be produced within days of primary sample collection 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.

P541

Exploring the role of a combined anti-MicroRNA-21 and TIMP3 mRNA nanotherapy interplay in modulating TIMP-3 expression: Implications for aortic aneurysm and cancer progression

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The field of RNA therapies has generated considerable interest in utilizing any type of exogenous nucleic acids transfection as a promising therapeutic approach. Tissue inhibitor of metalloproteinase 3 (TIMP-3) protein plays a crucial role in inhibiting matrix metalloproteinases (MMPs) and maintaining the integrity of the extracellular matrix (ECM). Decreased TIMP-3 expression has been associated with the progression of a huge variety of disease, specially aortic aneurysm and metastasis in cancer. Thus, increasing TIMP-3 expression could be a promising approach to treat such diseases. In this context, this study aims to investigate the effectiveness of TIMP-3 encoding mRNA transfection in modulating TIMP-3 protein expression. Oligopeptide end-modified poly (beta-amino ester) (pBAE) polyplexes are employed to protect and efficiently deliver mRNA to target cells. Additionally, we examine the interplay between exogenous transfection of microRNAs within the same biological pathway, specifically a anti-miR-21, with previously described TIMP-3 enhancer activity, in combination with TIMP-3 mRNA in engineering a more potent therapy targeting two aberrant mechanisms related with TIMP-3 expression

Our findings demonstrate the potential of our pBAE nanoparticles to efficiently transfect mRNA and to restore the balance between MMPs and TIMP-3 in muscle, cancer, and endothelial cells. Furthermore, the co-transfection of anti-miR-21, along with exogenous mRNA, enhances TIMP-3 expression. This discovery holds promise for attenuating disease progression in aortic aneurysm as well as reducing metastatic potential of a wide range of cancer types. Thus, our pBAE nanoparticles are confirmed as potential carriers for combined gene therapy applications.

P542

Novel glioma models and platforms for therapeutical and biomarker studies

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Cancer is one of the leading causes of death worldwide among people under the age of 70. Regular treatment of cancer includes surgery, chemotherapy and radiation therapy. Although combinations of different therapy modalities are possible, certain cancers like glioblastomas, the most common and devastating form of primary brain tumor in adults, have remained incurable. This pinpoints the need of more effective and additional therapies as well as more predictive experimental models to test them. We have collected here fresh samples from adult patients featuring diffusely infiltrating gliomas from grade 2 to 4 in order to investigate biomarkers, to develop models based on patient derived cultures and to examine their sensitivity to therapies. Characterization of parental tumors and cultures was carried out

immunohistochemically and immunofluorescence staining, respectively, whereas ability of oncolytic adenoviruses of 3 human serotypes to infect and kill cells were compared in glioma cultures. Quality and amount of tumor tissue were important factors for downstream studies. So far we have established a collection of low-passage patient derived cultures (in total > 30) that have origin of glioblastomas as well as lower grade gliomas (oligoastrocytomas, astrocytomas and oligodendrocytomas). From some patients different variants of cultures have been established. Among cell specific differentiation markers glial fibrillary acidic protein was strongly expressed in parental tumors and derived cultures. Heterogeneity was obvious in the expression of proliferation marker Ki-67 in tumors and growth of cultures. Expression of adenovirus 5 receptor CAR was low among samples. Expression of adenovirus 11p receptor CD46 was higher than CAR and it was maintained in cultures as well. Adenovirus 3 receptor DSG-2 was the most abundant adenovirus receptor in parental tissues but levels were reduced in cultures. Oncolytic potency of adenovirus 5 was comparable to adenovirus 11p while adenovirus 3 showed the poorest ability to kill glioma cells in vitro when equal multiplicity of infection was used. Thus levels of adenovirus receptors on glioma derived cultures did not correlate with infection efficacy in these models suggesting that they may not be a relevant indicator of clinical oncolytic potency. Further since none of the adenovirus receptors was predominant on a specific type of glioma, outcome might be improved with combination treatments and predictability using more advanced models latter of which are in progress.

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Expanding the Adenoviral treatment options for head and neck squamous cell carcinoma

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Head and neck squamous cell carcinomas (HNSCC) are hard to treat and difficult to resect. These tumors are amenable to adenoviral oncolytic tumor virotherapy because adenoviral vectors (AdV) can proliferate tumor-specifically in these cells. However, AdV vectors are mostly based on AdV serotype 5, which is limited in its applicability for in vivo treatment due to pre-existing immunity in the human population and its unfavorable liver tropism.

To identify new, improved personalized future AdV vectors for HNSCC treatment, we investigated the potential of naturally occurring human adenovirus serotypes to infect and possibly kill HNSCC cells in vitro. HNSCC cell lines were infected with a series of reporter gene-expressing adenoviruses resembling the natural diversity of human AdVs. Based on reporter gene expression, suitable serotypes were selected for further analyses. To investigate gene transfer efficiency and gene expression of these selected serotypes in a system that is more close to the patient, we established primary HNSCC tumor cell cultures from dissected tumors by measuring luciferase activity and quantifying GFP fluorescence, and the ability of the adenoviruses to undergo replication and lyse the host cells. Primary HNSCC cells were successfully cultured and propagated and characterized in terms of AdV receptor expression HPV status Upon transfection with different reporter gene expressing AdV serotypes different gene transfer as well as gene expression efficiencies were observed in the cultured primary HNCC tumor cells from each patient. In addition, oncolytic potential and cytotoxicity of the analyzed AdV serotypes differs from patient to patient.

Adenoviral vectors represent a promising therapeutic option for head and neck cancer. Optimization of adenoviral vectors using other serotypes is expected to potentially expand treatment options for HNSCC using adenoviral tumortherapy or oncolytic therapy avoiding preexisting immunity and preferred liver tropism of existing vectors. By expanding the options for oncolytic therapy of HNSCC, we hope to enable integrative and personalized treatment of individual patient tumors.

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Therapeutic efficacy of prodrug-activator gene therapy by retroviral replicating vectors for canine cancers

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The average lifespan of canines has significantly increased in recent years, and, like in humans, the prevalence of malignant tumors is rising. Retroviral Replicating Vectors (RRVs) have the ability to specifically infect cancer cells and then spread effectively throughout the tumor. RRVs themselves are not cytotoxic, but by carrying a prodrug-activator gene, tumor cell death can be induced by the administration of the prodrug. We have previously demonstrated that RRVs have excellent intratumor spread and antitumor effects in a variety of mouse models of human cancer. Human phase III clinical trials of RRV-mediated prodrug activator gene therapy using yeast cytosine deaminase (RRV-yCD) and 5-fluorocytosine (5-FC) prodrug demonstrated significantly prolonged survival in patients with two or more recurrences. We, therefore, hypothesized that RRVs might be effective against canine malignancies, and in this study, we examined the effectiveness of prodrug-activator gene therapy using two RRVs of different origins against canine cancers.

In the study of infection and spread efficiency using GFP-expressing RRVs, no infection spread was seen in normal canine cells, but efficient infection and spread of both RRVs were seen in all 10 canine tumor cell lines, particularly in fibrosarcoma cells, with infection efficiency of more than 90% by 10 days post-infection. Cell-killing assays using yCD-expressing RRVs, revealed no cell-killing effect in normal cells, but in all canine tumor cell lines tested, cell death was observed in response to the amount of the prodrug 5-FC, consistent with the earlier findings on RRV infection and spread efficiency. Using in vivo imaging, luciferase-expressing RRVs were seen to effectively propagate and spread throughout the tumor after intratumor administration in a mouse model of canine subcutaneous tumors. In treatment experiments in which yCD-expressing RRVs were administered intratumorally to a canine subcutaneous tumor mouse model, significant tumor growth inhibition was observed in both RRV treatment groups following 5-FC administration.

These data suggest that prodrug-activator gene therapy by RRVs appears to be useful in the treatment of canine cancers as well as human cancers.

P551

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 inherited lysosomal storage disorder (LSDs) with an autosomal recessive inheritance (1:500,000) caused by mutations in the *MAN2B1* gene encoding for the lysosomal α-D-mannosidase (MAN2B), which leads to a limited expression and consequently to a reduced activity of the corresponding enzymes. The lack or deficiency of lysosomal MAN2B results in the multisystemic 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 (ERT) and allogeneic haematopoietic stem cell transplantation (HSCT) from healthy donor (HD) are currently available treatments for α-MAN patients. However, many concerns remain about the effectiveness of these therapies in correcting the skeletal manifestations of the disease.
Ex-vivo lentiviral (LV)-based Hematopoietic Stem/progenitor Cells (HSPC)-gene therapy (GT) proved its efficacy in correcting the clinical phenotype of other LSDs, including Metachromati Leukodystrophy and Mucopolysaccharidosis type I Hurler, restoring the enzymatic activity also in non-hematopoietic cells in the diseased 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 show that human HD-derived CD34+ cells transduced with both WT and OPT LV-MAN2B retain their clonogenic and proliferation capability *in vitro*, reaching an average VCN of 1 in the liquid culture. LV-MAN2B transduced CD34+ cells as well as osteoclasts differentiated from transduced CD34+ cells show 2.8-fold and 2-fold increased intracellular and extracellular enzymatic activity, respectively, as compared to untransduced control. The released enzyme in the conditioned media was also capable to restore MAN2B activity in αMAN patients-derived fibroblasts at levels comparable to HD-fibroblasts, suggesting the cross-correction of non-hematopoietic cells in the presence of supra-physiological enzyme levels. By transplanting human CD34+ cells transduced with LV-MAN2B WT in immunodeficient mice, we observed multilineage reconstitution, with maintenance of human HSPC in murine BM at 12 weeks after transplantation. Moreover, mice transplanted with transduced cells displayed 3-fold higher enzyme activity in murine bone marrow than mock controls. We are currently characterizing the skeletal and immunological phenotype of a *MAN2B1-/-* mouse model, that will be instrumental for the proof-of-concept pre-clinical studies of HSPC-GT for α-Mannosidosis.
Altogether, our *in vitro* and *in vivo* results show that *MAN2B1* gene-transfer is well tolerated by human CD34+ cells, increasing the availability of extracellular MAN2B for cross-correcting

non-hematopoietic cells. Overall, these data support the development of an *ex-vivo* LV-based HSPC-gene therapy approach for the treatment of α-Mannosidosis.

P552

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 cilia beating with genetically corrected iPSC differentiated into air-liquid interface bronchial epithelium model (iALI).

Our project aims to evaluate the ability of a corrected iPSC cell line to functionally repair pathological models *in-vitro*. The differentiation of iPSC in iALI is well established within the team. We already generated a PCD patient iPSC cell line reprogrammed using Sendai viruses, and the corresponding CRISPR/Cas9 corrected cell line, as well as a wild type iPSC line and its CRISPR/Cas9 mutated counterpart. We also generated a GFP-iPSC cell line expressing the fluorescent GFP protein under the human elongation factor 1 alpha promoter (EF1a), allowing us to study the engraftment ability of GFP bronchial stem cells on a control epithelium model.

One main issue is to identify the competent cell type for regeneration of the adult bronchial epithelium. Indeed, there are several cell types constituting the bronchial epithelium, as well as several developmentally bronchial progenitor cells that could be considered. Our iALI differentiation process mimics the embryonic development and thus the iALI model may provide any cell type from the definitive endoderm to the mature bronchial epithelium. Our results suggest that lung progenitors at the ventralized anterior foregut endoderm stage, could be the most efficient cells for engraftment. Besides, 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. The second issue for bronchial epithelium cell replace would be to determine the best strategy to erode the bronchi prior to cell therapy. Such an erosion is considered 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. To this end, we compared different mechanical, chemical and enzymatic erosion strategies on the iALI model. Our results suggest a better efficiency with enzymatic erosion, showing a homogeneous detachment of the cells and a better engraftment of cells from the GFP-iPSC line.

In conclusion, engraftment of corrected lung progenitors to enzymatically eroded bronchial epithelium seems to be a promising therapeutic strategy to treat PCD. We need to refine in future experiments the best condition concerning enzymatic solution (concentration, time exposure) and graft cells number to assure functional recovery of the muco-ciliary clearance.

P553

Adeno-associated virus-mediated gene therapy for knee osteoarthritis – A phase I clinical trial report

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Knee osteoarthritis (OA) is a painful and degenerative joint disease, causing disability in millions. Only palliative treatments, which do not reverse joint damage, are available. Interleukin 1 (IL-1) is an inflammatory and catabolic cytokine that is a major contributor to the pathophysiology of OA, including pain signaling and tissue degeneration. Due to the rapid clearance from joints, sustained efficacy of small molecule drugs and biologics delivered locally, presents a significant challenge. To overcome this barrier, we developed a gene therapy, GNSC-001, that confers long-term IL-1 inhibition following a single intra-articular injection to the affected joint. GNSC-001 is a recombinant adeno-associated virus, serotype 2.5 (rAAV2.5) that encodes the human interleukin-1 receptor antagonist (IL-1Ra), a potent inhibitor of IL-1. Following pre-clinical pharmacology and toxicology studies, we initiated a Phase I clinical trial in subjects with knee OA.

The Phase I trial was an open-label, dose-escalation study to assess the safety and tolerability of GNSC-001 delivered as a single intra-articular injection into human knees with moderate OA (Kellgren Lawrence Grade 2-3). Subjects received 1×10^{11} vector genomes (vg)/knee (low-dose), 1×10^{12} vg/knee (mid-dose), or 1×10^{13} vg/knee (high-dose). Subjects underwent clinical evaluation at several time-points up to 12-months after GNSC-001 administration.

In this Phase I trial, GNSC-001 met the primary safety endpoint with no dose-limiting toxicity up to the 1×10^{13} vg high-dose. Mid- and high-doses resulted in synovial fluid IL-1Ra levels that remained above baseline throughout the trial. Less than 1% of viral genomes were detected in the blood 24-hours after dosing and were undetectable by 1-month. As expected, most of the subjects in the mid- and high-dose groups showed an increase in neutralizing antibodies to AAV2.5. However, a capsid targeted T-cell response was not detected in these dose groups. Subjects showed a trend toward improvement in pain and function, and none of them got worse.

P554

Clinical effect of intradiscal treatment with allogeneic mesenchymal stem cells derived from umbilical cord Wharton's jelly (WJ-MSC) in adults with Degenerative Disc Disease (DDD)

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In previous pre-clinical and clinical studies using regenerative therapies, mesenchymal stem cells (MSC) have been shown to promote a biological repairment process into the discal matrix in degenerative disc disease (DDD).

A retrospective cohort study analyzed the effects following MSC therapy based on MSC from human umbilical cord Wharton's jelly (WJ-MSC) in DDD patients. Ethical approval was obtained by an independent ethics committee (CEI-0324-07-2022). Clinical outcomes were measured by Visual-Analog-Scale (VAS), and Oswestry-Disability-Index (ODI). Clinical assessments were performed at baseline and 3-6-12 months after intradiscal/into-facet delivery of a single dose of $10x10^6$ WJ-MSC per target disc. Previously, allogeneic WJ-MSC were expanded in a culture medium supplemented with 10% human platelet lysate (hPL) up to passage 7. Cell marker expression and in vitro differentiation to mesodermal lineage were verified, as well as microbiological tests.

Twenty-five patients were treated between November/2021 to June/2022. All subjects completed 12 months of follow-up. The median time between the probable onset of symptomatic DDD and WJ-MSC therapy was 144 months (IQR=127). Eighty percent were male (n=20). The mean age was 45.12 years old (SD=12.90). The mean BMI was 26 kg/m^2 (SD=3.82). Lumbosacral discopathy was confirmed in 18 patients (72%), and thoracic discopathy in 7 (28%). The most common number of affected discs was 3 (n=10/40%). In the pre-therapy spine MRI, 6 patients showed MODIC-1 changes (24%) and 7 MODIC-2 changes (28%). Fifteen were classified as Pfirrmann-III (60%) and 4 as Pfirrmann-IV (16%). Seven subjects (28%) had undergone one or more previous spinal surgeries. Fourteen had a history of radiculopathy (56%).

The median pre-therapy VAS score was 4 (IQR=4), and the median VAS-12m score was 2 (IQR=4) (*p=0.007*). In 19 subjects, the VAS-12m score improved or remained the same compared to pre-therapy (76%) (*p=0.003*). The median pre-therapy ODI was 24% (IQR=16), and at 12 months, it was 16% (IQR=26) (*p=0.130*), however, in 20 subjects, the ODI-12m score improved or remained the same compared to pre-therapy (80%) (*p=0.001*).

Regarding the evaluated outcome, 8 patients responded favorably to WJ-MSC (32%) (a responder was defined as a patient with a reduction in ODI percentage >=10% when comparing pre-therapy scores with the 12-month follow-up). However, an additional 4 subjects responded to therapy exclusively based on improvement in VAS (16%), but not in ODI. The Log-Rank test showed significant differences (at a 90% confidence level) as follow: Responder to therapy based on ODI (yes/no) (*p=0.003*), number of affected discs (*p=0.006*), number of discs receiving WJ-MSC injections (*p=0.004*), reduction in VAS from pre-therapy to VAS-12m (worsened vs improved or remained the same) (*p=0.047*), MODIC-1 (no or indeterminate vs yes) (p=0.075), MODIC-2 (no or indeterminate vs yes) (*p=0.076*). A Cox regression analysis revealed that the significant predictors were: MODIC-1 (yes) (*p=0.047*, HR_a*=5.38 95%CI=1.00,29.07*, *Proportional-Hazard p-value=0.581*), number of affected discs (*p=0.043*, HR_a*=0.26 95%CI=0.07,0.94*, *Proportional-Hazard p-value=0.171*). The final model showed moderate fit (*LR p=0.088*, *AIC=37.99*, R^2*=56.31%*). No serious adverse events were reported.

We conclude that intradiscal WJ-MSC delivery was safe and showed relevant clinical effects in DDD patients.

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Clinical effect of intraarticular treatment with allogeneic mesenchymal stem cells derived from umbilical cord Wharton's jelly (WJ-MSC) in adults with Knee Osteoarthritis (KOA)

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Ortho-biological regenerative therapies such as mesenchymal stem cells (MSC) have been shown to promote cartilage biological repairment in knee osteoarthritis (KOA).

A retrospective cohort study analyzed the effects following therapy based on MSC from human umbilical cord Wharton's jelly (WJ-MSC) in KOA patients. Ethical approval was obtained by an independent ethics committee (CEI-0435-11-2022). Clinical outcomes were measured by the Short-Form12 questionnaire, Visual-Analog-Scale (VAS), and Western-Ontario-McMaster-Index (WOMAC). Clinical assessments were performed at baseline and six months after intra-articular delivery of a single dose of $40x10^6$ WJ-MSC per target knee. Previously, allogeneic WJ-MSC were expanded in a culture medium supplemented with 10% human platelet lysate (hPL) up to passage 7. Cell marker expression and in vitro differentiation to mesodermal lineage were verified, as well as microbiological tests.

Thirty-five knees (19 patients) were treated between February/2020 to October/2022. All subjects completed 6 months of follow-up. The median time between probable KOA diagnosis or onset and WJ-MSC therapy was 184.4 months (IQR=127.02). Eighteen patients were male (94.7%). The median age was 59 (IQR=18). The KOA radiographical status (Kellgren-Lawrence) was classified as mild (n=16/46%), moderate (n=14/40%), and severe (n=5/14%).

The median pre-therapy VAS score was 5 (IQR=2), and the 6-month was 4 (IQR=2.5) (*p<0.001*). Thirty-one patients (89%) did not experience VAS worsening compared to baseline (*p<0.001*). The median pre-therapy WOMAC-general score was 37 (IQR=26.5), and the 6-month was 27 (IQR=18.5) (*p=0.022*). The median pre-therapy WOMAC-pain score was 7 (IQR=5), and post-therapy was 4 (IQR=5) (*p<0.001*). The median baseline WOMAC-functionality score was 24 (IQR=20.5), and post-therapy was 21 (IQR=13) (*p=0.045*). Regarding the WOMAC-stiffness score, there were no significant differences between baseline and six months medians (*p=0.839*). The median baseline SF12-mental score was 52.38 (IQR=57.14), and the 6-month was 64.28 (IQR=42.85) (*p=0.003*). Changes in SF12-physical score were not significant statistically (*p=0.971*). However, after six months, the global SF12 improved or at least remained the same compared with the baseline measurement in 14 patients (78%) (*p=0.009*).

The responder status (yes/no) to therapy was the outcome (a target knee that achieved a WOMAC-general score difference >=20% between baseline and post-treatment). Sixteen knees responded to the therapy (46%). The Log-Rank test showed significant differences in

Age (<=58 vs >=59) (*p=0.009*), BMI (normal/overweight/obesity) (*p=0.018*), patellofemoral-KOA (*p=0.041*), partial-arthroplasty (*p=0.012*), total-arthroplasty (*p=0.001*), previous cartilage repair (*p=0.043*), physical therapy (*p=0.003*), 6-month VAS (>=5 vs <=4) (*p=0.038*), reduction in 6-month VAS (same or improved vs worsened) (*p=0.039*), responder to

therapy based on pain improvement (*p=0.002*) and responder based on functionality improvement (*p=0.011*).

The only significant predictors in multivariate analysis were patellofemoral-KOA (*p=0.004*, HR_a*=58.49*, *95%CI=4.49-762.00*) and responder to therapy based on pain improvement (*p=0.007*, HR_a*=36.70*, *95%CI=6-302.00*). The final model demonstrated a good fit (Likelihood *p=0.005*, Wald-test *p=0.013*, AIC=71.6, R^2=82.17%). The assumption of Cox proportional hazards was met (*p=0.061*). No significant confounding effect was observed when adjusting for prior use of biological therapies (MSC/PRP/HA) and prior treatments (NSAID/corticosteroids/physical therapy).

Nine patients (26%) reported some adverse events, none of which were serious. We conclude that intraarticular WJ-MSC delivery was safe and showed relevant clinical effects in KOA patients.

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A carbohydrate sulfotransferase mutant zebrafish shows importance of keratan sulfate proteoglycan in skeletal structure

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Proteoglycans (PGs), a large class of highly glycosylated proteins, contain side chains of glycosaminoglycans (GAGs) that are covalently bonded to each other. These macromolecules can have different GAG compositions and they can be found in the extracellular matrix, on the cell surface, or intracellularly. Various proteoglycans are known to have roles including regulation of extracellular matrix (ECM) via their interaction with collagens, cell-to-cell signaling, and chemokine diffusion. Both the protein cores and the GAG side chains of PGs interact with other molecules and modulate functions of these complex molecules. Sulfation of the GAGs are essential for the functions of PGs. Both bone and cartilage ECM contains proteoglycans of chondroitin / dermatan sulfate (CS/DS) and keratan sulfate family and defects in the carbohydrate sulfotransferase (CHST) genes are associated with rare skeletal diseases. In this study zebrafish model organism was used to investigate importance of KSPGs in cartilage and bone development. To this end, KSPG specific chst6 gene was mutated via CRISPR/Cas9-mediated gene editing. Several loss-of-function mutant alleles were obtained. Inhibition of keratan sulfate proteoglycan was confirmed with ELISA and immunofluorescence staining. Mutant embryos had defects in trunk formation with varying severity: pericardial edema, short body length, and body curvation. Most severe mutants had tail truncation or folding and did not survive beyond 5 days. This phenotype was fully rescued by mRNA injection. Mild mutants showed slower response and swirling swimming pattern when stimulated by touch. Lower jaw cartilage was stained with alcian blue, which showed altered jaw and joint development. Mutants with mild phenotype survived into adulthood, and the bone structure was examined with alizarin red staining. Curving of the spinal cord (mild scoliosis) and defects in vertebrae were detected in adults as of 8 months of age. There is limited information on KSPG-related skeletal diseases in the literature, and this study shows that sulfation of KSPGs is essential for the proper development of skeletal tissues.

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Increased expression or activation of TRPML1 reduces hepatic storage of toxic Z alpha-1 antitrypsin

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Mutant Z-alpha-1 antitrypsin (ATZ) accumulates in the liver and is the prototype of proteotoxic hepatic disease. Strategies aiming at clearance of polymeric ATZ have therapeutic potential. Transient receptor potential mucolipin 1 (TRPML1) is a lysosomal calcium channel that maintains lysosomal homeostasis. In this study, we investigated whether increasing lysosomal exocytosis by *TRPML1* gene transfer or small molecule-mediated activation of TRPML1 reduce hepatic ATZ globules and fibrosis in PiZ transgenic mice, that express the human ATZ forming Periodic acid–Schiff (PAS)-positive globules in the liver. By intravenous injections, PiZ mice received an AAV8 vector expressing the human TRPML1 under the control of the hepatocyte-specific thyroxin-binding globulin (TBG) promoter (AAV-TRPML1) and were sacrificed 12 weeks post-injection. Compared to AAV-GFP-injected controls, livers of PiZ mice injected with AAV-TRPML1 showed a significant reduction in PAS-positive globules and decreased liver fibrosis. Liver immunofluorescence with an antibody recognizing ATZ polymers showed a decrease in number and size of ATZ polymers and the immunoblotting on soluble and insoluble fractions confirmed the reduction of ATZ polymers. By 2 weeks post-injection, PiZ mice injected with AAV-TRPML1 also showed an increase of circulating ATZ, suggesting increased secretion. Translocation of lysosomes to the plasma membrane is the hallmark of lysosomal exocytosis and by electron microscopy increased number of lysosomes close to the plasma membrane was detected in liver cells of PiZ mice injected with AAV-TRPML1 compared to controls. Moreover, increased activities of the lysosomal enzymes iduronate sulfatase (IDS) and galactosamine (N-acetyl)-6-sulfatase (GALNS) were detected in sera of AAV-TRPML1–injected PiZ mice compared to controls. PiZ mice receiving daily intraperitoneal injections of the TRPML-1 activator ML-SA5 also showed reduced hepatic PAS-positive globules and soluble and insoluble AAT fractions, and transient increase of serum ATZ polymers. In conclusion, our results show that targeting TRPML1 and lysosomal exocytosis is a novel approach for treatment of the liver disease due to ATZ and potentially other diseases due to proteotoxic liver storage.

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Intra-CSF AAV-FGF21 gene therapy reverts type 2 diabetes, obesity and cognitive decline

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Type 2 Diabetes (TD2) and obesity are closely related diseases with an increasing worldwide prevalence. Moreover, TD2 and obese patients have high risk of developing dementia and cognitive decline in the long-term, alteration that can neither be cured nor prevented with conventional treatments. Fibroblast growth factor 21 (FGF21) has become a promising

therapeutic agent for the treatment of obesity and T2D and it can also act in different brain regions to regulate whole body metabolism. Here, we found that intra-cerebrospinal fluid (CSF) administration of AAV-FGF21 vectors to obese and insulin resistant mice resulted in specific overexpression of FGF21 in the CNS, without an increase in the FGF-21 circulating levels. Long-term brain FGF21 expression resulted in reduction of body weight gain, adipose tissue inflammation, hepatic steatosis and fibrosis and counteraction of insulin resistance. These metabolic improvements were parallel to enhanced energy expenditure, most likely induced by sympathetic outflow, that activated non-shivering thermogenesis in brown adipose tissue and induced browning of subcutaneous white adipose tissue. In addition, treatment with intra-CSF AAV-FGF21 gene therapy prevented memory loss, improved learning capacity and decreased anxiety-like behaviour, key features of advanced T2D. Altogether, these results point out the potential of AAV-mediated gene transfer of FGF21 to the CNS to treat obesity, T2D and associated neurodegenerative disorders.

P559

Single AAV gene therapy with mini-Glycogen Debranching Enzyme for glycogen storage disease type III

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Glycogen storage disease type III (GSDIII) is a rare metabolic disorder that affects the liver, skeletal muscles, and heart. It is caused by mutations in the AGL gene, which encodes the glycogen debranching enzyme (GDE). However, using the full-length GDE cDNA in a single adeno-associated virus (AAV) vector strategy poses challenges due to its large size (4.6 kb). Alternative approaches, such as dual vectors or bacterial orthologs of GDE, have been proposed but they face obstacles related to immune responses and higher required doses for clinical translation.

Here, we developed a gene therapy strategy for GSDIII using a truncated version of the GDE enzyme delivered by a single rAAV vector to correct skeletal muscles and heart impairment in *Agl*$^{-/-}$ mice and rats and to decrease glycogen content in skeletal myotubes differentiated from Agl$^{-/-}$ human pluripotent stem cells (iPSC). By employing molecular modelling and known AGL missense mutations, we identified the N-terminal region of GDE as a promising target for size reduction while preserving its activity. Administration of mini-GDE ΔNter2-expressing rAAV vectors via intravenous injection in adult Agl$^{-/-}$ mice and rats resulted in complete glycogen clearance in muscles and heart, leading to the normalization of histology features three months after treatment. We also evaluated the serum levels of myomesin-3 (Myom3) fragments, a known biomarker of muscle dystrophies that was also found elevated in Agl$^{-/-}$ mice and rats at baseline. Myom3 levels were normalized three months after treatment in both models correlating with complete reversion of muscle strength impairment in Agl$^{-/-}$ mice. Furthermore, transduction of Agl$^{-/-}$ iPSC-derived muscle cells with mini-GDE ΔNter2-expressing rAAV vectors nearly eliminated glycogen accumulation without any evident toxicity compared to mock-transduced cells. Importantly, the mini-GDE ΔNter2 exhibited comparable glycogen clearance to the full-length

GDE in vivo. Additionally, its reduced size greatly improved the yields and quality of the encapsidated rAAV vector.

In summary, our findings demonstrate, for the first time, the efficacy of a functional truncated GDE transgene in correcting the muscle and heart phenotype in both rodents and human models of GSDIII. These results support the clinical application of this approach in GSDIII patients.

P560

Interim non-neurological, non-skeletal outcomes after hematopoietic stem and progenitor cell-gene therapy for Mucopolysaccharidosis type I Hurler

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Mucopolysaccharidosis type I Hurler (MPS-IH) is a lysosomal storage disease caused by deficiency of α-L-iduronidase, resulting in accumulation of glycosaminoglycans in multiple organs, including eyes, ears, heart, musculoskeletal and central nervous systems. Despite successful allogeneic haematopoietic stem cell transplantation (HSCT), MPS-IH patients experience residual disease burden at multiple organs, affecting quality-of-life.

In a phase I/II study (NCT03488394), *ex-vivo* hematopoietic stem and progenitors cells (HSPC)-gene therapy (GT) demonstrated extensive metabolic correction, however its effects on multiple organs is still under investigation.

Eight MPS-IH patients (6M, 2F; mean age ±SD at treatment: 1.9±0.5 years) received HSPC-GT (Gentner et al. N Engl J Med. 2021) and were followed-up for median of 3 years after treatment. Corneal clouding and adenoid/tonsillar hypertrophy were assessed with subjective clinical grading scores, while auditory function with brainstorm auditory evoked responses. Cardiac abnormalities were assessed by echocardiography. Carpal tunnel was evaluated with electroneurography (ENG) and median nerve ultrasound. Baseline results were compared with those obtained after treatment.

While at baseline all subjects had evidence of infiltration or clouding of the cornea, patients improved (75%) or remained stable (25%) at last follow-up. Three patients underwent tonsillectomy and/or adenoidectomy after HSPC-GT. Most patients show mild to severe peripheral hearing loss but only one temporarily required hearing aids. None developed cardiomyopathy or severe valvular heart disease. One patient received carpal tunnel surgery before HSPC-GT, while another patient underwent surgery 6 month after HSPC-GT due to severe carpal tunnel syndrome already present at baseline. All the others showed stable nerve conduction after treatment.

A longer follow-up is needed to draw definitive conclusions on the effects of HSPC-GT on these clinical outcomes.

P561

AAV-FGF21 gene therapy extends healthspan in old mice

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Aging is the primary risk factor for major human pathologies including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases. This deteriorative process is also accompanied by major changes in body composition that lead to an increase in body weight and insulin resistance. As population ages, obesity, diabetes and dementia are three related conditions that are increasing in prevalence and share common pathways. We have previously shown that intramuscular (IM) administration of AAV1 vectors encoding FGF21 (AAV1-FGF21) in young healthy mice prevented the increase in body weight gain, adiposity and insulin resistance associated with aging. Here, we demonstrated that IM administration of AAV1-FGF21 in aged mice (>12 months old) mediated long-lasting increase in FGF21 circulating levels, which extended health and life span and reverted age-related insulin resistance, body weight gain, and neuromuscular and cognitive decline. Moreover, this treatment also modulated gene expression in major relevant metabolic organs as well as in the brain. These results underscore the potential of FGF21 gene therapy to promote healthy aging. Moreover, all these results further highlight the safety of the skeletal muscle-directed AAV1-FGF21 gene therapy.

P562

Modelling parameters for optimal AAV vector-mediated liver gene transfer and gene editing in neonatal, infantile and juvenile age

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Recombinant adeno-associated virus (AAV) vectors are well-recognized gene delivery systems for gene replacement therapy of monogenic liver disorders, having excellent benefit-risk ratio as demonstrated in clinical trials for hemophilia and other diseases in adult subjects.

However, liver maturation plays an important role in the efficiency, durability and safety of AAV-mediate gene correction in pediatric population. In fact, in traditional gene transfer gene therapy, the therapeutic payload remains mostly episomal, raising limitations when applied during infancy or juvenile age due to vector loss associated to hepatocyte duplication during liver

growth. Conversely, gene editing and genome targeting strategies have shown promising potential to permanently correct the diseased genome when applied during neonatal and infantile age. The required rate of correction varies depending on both the disease type and the presence or absence of growth advantage of the edited cells.

Thus, the identification of key parameters driving the efficiency and duration of AAV-mediated gene correction (whether gene therapy or genome editing) in pediatric subjects requires further definition before its translation to the clinic.

As the developmental state of the liver is a critical parameter for the effective and long-lasting gene therapy and genome editing, the proliferative and pluripotency profile of the liver was assessed at the different ages selected for vector administration (P4, P11 and P18) and compared to the mature liver (P30, P60 and P120) in mice. Active proliferation in post-natal liver was determined using the cycle track system, which relies on the cell-cycle specific activation of Cre recombinase that in turn activates a reporter gene (GFP or mCherry) only in duplicating hepatocytes. The system well correlates with known markers of cell cycle such as Ki67 and PH3, as well as well-established detection method based on bromodeoxyuridine (BrdU) or 5-ethynyl-2'-deoxyuridine (EdU) incorporation. The cycle track system and EdU incorporation were also coupled with the GeneRide/CRISPR/Cas9 platform to study the interplay between cell cycle and HDR at different post-natal ages in neonatal (P1 and P3), infantile (P9) and juvenile (P16) livers.

Finally, several genes involved in liver proliferation, pluripotency and maturation (polyipolidization) were evaluated in mouse livers at different ages to profile predictive molecular markers of liver maturation. Those markers were next validated and correlated in mRNA preparations and liver biopsies derived from human subjects at different ages (2, 5 10, 15, 20, and 30 years of age).

In conclusion, our work provides guidance for the translation of AAV vector-mediated gene transfer and genome editing for liver to the pediatric patient population.

P563

Systemic delivery of rAAVSNY001-h*PAH* mediates persistent phenotypical correction of Phenylketonuria disease in *Pah*-KO mice

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Phenylketonuria (PKU) is a genetic deficiency of phenylalanine hydroxylase (PAH) in liver and results in elevated levels of phenylalanine (Phe) in blood and brain with subsequent neurotoxicity. Standard care consists of Phe restricted diet but adherence among teen and adult patients is poor. In addition, alternative therapies are only effective in a small percent of patients. To address the disease at its root cause, Sanofi is developing a recombinant adeno-associated virus (rAAV) based gene therapy to restore liver PAH activity.

A novel AAV capsid - SNY001 was selected due to its superior liver transduction potency in non-human primates compared to other commonly used AAV capsids. GLP grade rAAVSNY001-h*PAH* vector was evaluated in *Pah*-KO mice for its disease correction efficiency following a single intravenous administration at three dose levels. As a result of PAH activity

restoration (corresponding to 46%, 350% and 450% of heterozygous PAH levels, respectively), persistent normalization of blood Phe level was achieved in 100% of the animals in the two higher dose groups and 75% of the animals in the lowest dose group, and the persistent reduction of blood Phe was associated with stable vector copy numbers and hPAH-mRNA levels in liver as measured at 4 and 13-weeks post treatment. Furthermore, concomitant reduction of brain Phe, normalization of brain neurotransmitters and restoration of nest building ability were also observed in Pah-KO mice at all dose levels. Our work demonstrated that systemic treatment of rAAVSNY001-h*PAH* corrects PKU related pathologies in *Pah*-KO mice in a sustained manner and support further development of rAAVSNY001-h*PAH* for the treatment of PKU.

P564

Liver-directed gene targeting as a potential therapy for Fabry disease

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Fabry disease (FD) is an X-linked inherited, lysosomal storage disorder caused by mutations in the Alpha Galactosidase-A (GLA) gene. This gene encodes for the GLA enzyme which is responsible for the catabolism of glycosphingolipids like globotriaosylceramide (Gb3). Accumulation of Gb3 in lysosomes results in systemic clinical manifestations and reduced lifespan. Enzyme replacement therapy (ERT) and chaperone therapy are the available treatments for FD however, noncurative and with limitations.

We developed a potential therapeutic approach based on the permanent genetic modification of hepatocytes to express the GLA enzyme by targeting the albumin locus *in vivo*. To model late-onset and early-onset FD, we treated juvenile (P30) and neonatal (P5) Fabry mice with an AAV8 donor vector containing mAlb homology arms and a codon-optimised version of the human GLA cDNA. This treatment was coupled with the AAV-mediated delivery of the CRISPR/SaCas9 platform to increase targeting efficiency. Treatment of juvenile Fabry mice (donor, 3.0E13 vg/kg; SaCas9, 6.0E12 vg/kg) resulted in elevated GLA enzyme activity which was stable till the termination of the experiment at 5 months of age, accompanied by a 70-80% reduction in Lyso-Gb3 accumulation in liver, kidneys and heart, compared to untreated mutant mice. To increase safety of the procedure, concerns related to the use of programmable nucleases were avoided by applying a nuclease-free approach. Juvenile animals were treated only with the donor vector (3.0E13 vg/kg), coupled with the treatment with fludarabine, which enhances gene targeting rate. This nuclease-free strategy resulted in increased plasma GLA activity compared to donor-only treated mice, accompanied by 80-95% of lyso-Gb3 clearance.

When we treated neonatal P5 Fabry mice with donor and SaCas9 AAVs, the treatment was significantly more efficient than in juvenile animals due to the increased targeting rate observed in proliferating hepatocytes present in a growing liver. In fact, we were able to completely clear lyso-Gb3 in plasma and in the different organs with the highest dose of 3.0E14 vg/kg of donor vector, while a dose of 3.0E13 vg/kg resulted in a reduction of 95-98% in plasma and target organs.

A dose escalation study with AAV-mediated episomal gene therapy was also done as a proof-of-principle in juvenile Faby KO mice using a strong liver-specific promoter and the human GLA transgene. Animals treated at 3.0E12vg/kg and higher doses were able to reduce substrate

accumulation by 98-100% in plasma and target tissues. Treatment with the lowest dose of the AAV vector (3.0E11vg/kg) resulted in the clearance of 85-95% lyso-Gb3 in the bloodstream and tissues proving the efficacy of the treatment for late-onset FD. ERT-treated animals were considered for comparative evaluation of the treatment.

This data is inclined towards a promising one-shot therapy using a safer gene-editing approach to ameliorate the phenotype irrespective of the mutation, targeting both the early and late-onset FD conditions. To test the translational applicability of this integrative strategy, AAV-LK03 vectors containing human ALB homology arms have been tested in human liver cell lines and will be validated in primary cultures of human hepatocytes, and in humanized mice to generate consistent preclinical support.

P565

Mitochondrial base editing for gene therapy and modelling disease

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Around 1 in 10,000 people suffer from severe, hereditary mitochondrial disease. Current gene editing technologies are greatly accelerating genetic cures for diseases caused by mutations in nuclear DNA, but they offer no solutions for hereditary diseases that are caused by defects in mitochondrial DNA. Excitingly, a recent first proof-of-concept study showed that a novel gene editor called DdCBE enables mitochondrial DNA base editing in HEK293T cells. However, its efficiency in correcting pathogenic mitochondrial mutations in primary, patient-derived cells is unknown, let alone its capacity to restore mitochondrial function.

We therefore created the mitochondrial m.15150G>A mutation in human liver organoids, which introduces a premature stop codon in *MT-CYB* encoding for cytochrome B. We discovered high variability in m.15150G>A editing efficiency between individual organoids grown from single cells. In addition, we corrected the pathogenic m.4291T>C variant (affecting the mitochondrial Isoleucine tRNA) in patient-derived skin fibroblasts. Similar to the variability found in m.15150G>A organoids, the efficiency of m.4291C gene correction was highly variable between single fibroblast-derived clones. Furthermore, functional analysis demonstrated that correction of m.4291C led to a recovery in mitochondrial membrane potential and that the level of gene correction correlated with mitochondrial function.

These results demonstrate the potential of DdCBE to generate novel models for mitochondrial disease from patient-derived and disease-relevant tissue. They further indicate that the efficiency of mitochondrial gene editing is highly variable between individual *in vitro* cultured primary cells, which suggests that editing efficiency can be further enhanced to optimize the efficacy of future mitochondrial gene correction therapies. Furthermore, we show that gene correction of patient-derived fibroblasts improves mitochondrial membrane potential. Thus, mitochondrial gene editing is a valuable tool for developing breakthrough therapeutic strategies to the benefit of mitochondrial patients.

P566

Neonatal AAV gene transfer successfully treats severe maple syrup urine disease (MSUD) in mice

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Maple syrup urine disease (MSUD) is a rare recessively inherited metabolic disorder due to the dysfunction of the Branched-chain Keto acid Dehydrogenase (BCKD) enzyme, which leads to accumulation of branched chain amino acids causing neonatal death if untreated. MSUD is caused by mutations in any of the following 3 genes, *BCKDHA*, *BCKDHB* and *DBT*, coding for different subunit of BCKD. MSUD represents an unmet need as its current management is based on a life-long low-protein diet which, however, does not prevent the risk of acute decompensations and long-term neuropsychiatric defects. Orthotopic liver transplantation is a beneficial therapeutic option, which shows that restoration of only a fraction of whole-body BCKD enzyme activity is therapeutic. In this study, we developed an adeno-associated virus (AAV8) gene therapy for *BCKDHA* and *BCKDHB* genes to treat MSUD in mice.[1,2] We performed the first extensive characterization of *Bckdha*$^{-/-}$ and *Bckdhb*$^{-/-}$ mouse models, which exhibit a lethal neonatal phenotype faithfully mimicking human severe MSUD. Animals were treated at P0 with intravenous injections with human *BCKDHA* AAV8 vectors under the control of an ubiquitous or a liver-specific promoter for *Bckdha*$^{-/-}$ mice and with human *BCKDHB* AAV8 vectors under the control of an ubiquitous promoter for *Bckdhb*$^{-/-}$ mice. Treatment with vectors carrying the ubiquitous promoter achieved long-term (> 6 months) rescue in both MSUD models, while treatment with the vector carrying the liver-specific promoter led to partial, though sustained rescue in *Bckdha*$^{-/-}$ mice. We provided herein the proof-of-concept of the efficacy of gene therapy for MSUD, in *Bckdha*$^{-/-}$ and *Bckdhb*$^{-/-}$ mice, demonstrating its potential for clinical translation.

1. Pontoizeau, C. *et al.* Neonatal gene therapy achieves sustained disease rescue of maple syrup urine disease in mice. *Nat. Commun.* 13, 3278 (2022).

2. Pontoizeau, C. *et al.* Successful treatment of severe MSUD in *Bckdhb*$^{-/-}$ mice with neonatal AAV gene therapy. *J. Inherit. Metab. Dis.* (2023) doi:10.1002/jimd.12604.

P567

Therapeutic Efficacy of the Combination of Long-Term Penicillamine Treatment with a Single Administration of VTX-801 in Wilson's Disease Mice

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Wilson's disease (WD) is a rare disorder of copper metabolism inherited in an autosomal recessive manner. It is due to missense mutations in *ATP7B*, a copper-transporting P-type ATPase, that lead to hepatic, neurologic, or psychiatric symptoms. Left untreated, the condition progresses to severely debilitating complications and death. Current medical management relies on life-long oral treatment with copper chelators such as D-penicillamine (DPA) or with zinc salts.

VTX-801 is a recombinant adeno-associated vector (rAAV) carrying a shortened version of the human ATP7B gene. VTX-801 has demonstrated to provide long-term correction of copper metabolism and ceruloplasmin levels, preservation of liver integrity and function when administered at early stage of the disease in WD mice. Based on these results, GATEWAY, a phase I/II clinical trial is underway to evaluate VTX-801 in adult patients with WD.

We then evaluated the therapeutic effect of a single administration of VTX-801 in 20-week-old WD mice treated with DPA from 4 weeks of age. Efficacy was assessed through analysis of copper content in urine, blood and tissues, transaminases, hematology and histological analysis, including markers of inflammation and fibrosis at sacrifice, 24 weeks after VTX-801 injection.

All treatments (DPA only, VTX-801 only and DPA+VTX-801) reduced the levels of hepatic copper in WD mice. Nonetheless, only the groups of mice treated with VTX-801 displayed a significantly lower concentration of copper in liver (compared to untreated WD mice), with lowest levels in mice receiving the combination. The percentage of exchangeable to total serum copper (REC), that appreciates the toxic fraction of copper in blood, was high in DPA-treated mice but was normalized by VTX-801 treatment alone or in combination with DPA. Moreover, ceruloplasmin activity in circulation was restored only in VTX-801 treated animals. These results emphasize the strong de-coppering effect of the combination therapy with long-term DPA followed by a single administration of VTX-801. Importantly, no copper deficiency was observed in those mice.

Compared to the natural progression of liver pathology observed in WD mice, animals treated with VTX-801 showed a significant decrease in inflammatory cell focus/foci, diffuse hepatocyte hypertrophy and fibrosis, but also displayed increased steatosis. DPA-treated animals showed a normal liver parenchyma, when administered alone or in combination with VTX-801. When combined with DPA treatment, VTX-801 reduced severity score for all the histological parameters tested.

Altogether these results indicate that treatment with a copper chelator contributes to stabilization of liver disease progression in WD mice. DPA treatment significantly improved the therapeutic efficacy of VTX-801 when administered at 20 weeks of age resulting in full restoration of copper metabolism in the absence of adverse events.

P568

Preclinical development of an ex vivo gene therapy for Mucopolysaccharidosis type II

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Mucopolysaccharidosis type II (MPS II, Hunter syndrome) is an X-linked inherited disorder of carbohydrate metabolism. It is caused by mutations in the IDS gene, encoding the lysosomal enzyme iduronate-2-sulfatase (IDS). IDS deficiency causes glycosaminoglycan (GAG) accumulation in the lysosomes, leading to a severe impairment of cell metabolism and finally to cell death. The central nervous system (CNS) is particularly sensitive to this metabolic defect. MPS II causes distinctive facial features, visceral abnormalities and progressive intellectual decline, and the treatment is only symptomatic.

We designed an original gene therapy (GT) approach based on the autologous transplantation of hematopoietic stem/progenitor cells (HSPCs) genetically modified by lentiviral (LV) gene transfer to restore IDS expression. The GT approach was tested in vivo in pre-symptomatic and symptomatic MPS II mice. Experimental mice were monitored for survival, behavior and pathology hallmarks long-term. GT rescued behavioral, skeletal, biochemical and pathological disease-associated deficits and significantly increased animal survival, with all GT-treated mice out-surviving the control mock-transplanted mice. GT resulted in restoration of IDS activity up to 50% of the normal level and reduction of GAG accumulation in the brain of the treated animals. Safety monitoring of the treated mice did not show any treatment-related adverse events. Based on these results in the animal model of the disease, we assessed the GT strategy in a toxicology and biodistribution study in humanized immunodeficient NOD Scid Gamma (NSG) mice . The study employed human HSPCs transduced with large-scale produced Lentiviral vector (LVV) and two different manufacturing protocols intended for clinical use, which differ in the total duration of the LVV transduction, cell concentration and cytokine cocktail. Human HSPCs transduced with these protocols showed a preserved functionality in repopulated immunodeficient mice, as compared to mock-transduced cells, with a sustained engraftment up to 16 weeks post-transplant and multilineage differentiation. LVV profiling and in vitro immortalization assay revealed the absence of genotoxicity. Interestingly, we observed a robust engraftment in vivo of highly-transduced cells with respect to the infused cell products. Immunophenotypic analysis of the cell products manufactured with the two differential protocols revealed a relatively higher content of primitive HSCs in the cell products transduced with the shortest vs longest manufacturing process. This was associated with a higher transduction efficiency of long-term NSG-repopulating HSCs by the short protocol, as shown by the increased vector copy number observed in vivo as compared to the infused cell product. This effect, which was maintained when the cell manufacturing protocol was applied to a different LVV not expressing the IDS enzyme, is likely related to the cell culture conditions that favor the efficient transduction of primitive HSCs. Overall, the study will be instrumental to a rapid progression towards the clinical development of our GT strategy for MPS II.

P569

Exploring the effect of haematopoietic stem cell gene therapy (HSC-GT) pre-conditioning regimes on brain vasculature to identify novel targets to improve central nervous system-directed cellular engraftment for neurological lysosomal storage disease HSC-GT

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Lysosomal storage diseases (LSDs) are multisystem inherited metabolic disorders caused by dysfunctional lysosomal enzymes, resulting in the accumulation of undegraded macromolecules in a variety of organs/tissues, including the central nervous system (CNS). It is particularly difficult to treat CNS pathology due to the blood-brain barrier (BBB), a highly selective semi-permeable membrane which protects the brain from circulating toxins and pathogens, but consequently also limits the crossing of therapeutics into the CNS. Current treatment strategies include enzyme replacement therapy and haematopoietic stem/progenitor cell gene therapy (HSC-GT). In the last decade, HSC-GT has been partially effective in attenuating LSD patients' neurological symptoms; a subpopulation of HSCs can cross the BBB, engraft the damaged CNS as microglia, and secrete enzymes that are taken up by enzyme-deficient cells, hence preventing further neurodegeneration. One poorly understood but essential component of this process is the passage of HSCs across the BBB, which is elevated in busulfan-conditioned animals compared to other pre-conditioning regimes and results in higher levels of cellular engraftment in the CNS. Published literature provides evidence for clearance of the microglia as the underlying mechanism, and there is suggestion that vascular injury may also be implicated, although this has not been explored in depth. Here we present detailed immunofluorescent imaging and quantification of CNS vasculature *in vivo* at early (24 hour), mid (2-week) and late (5-week) post-conditioning timepoints. We assessed large-scale vascular structure in the CNS and their coverage with pericytes based on PECAM-1 (vessel) and PDGFRβ (pericyte) expression, as well as measuring intensity and localisation of endothelial tight junction proteins claudin-5, JAM-A, and ZO-1. These findings provide new insight into the effect of pre-conditioning regimes on the CNS vasculature, opening up new avenues of investigation for manipulation of the BBB, with a view to increasing permeability in order to increase HSC transmigration and thereby improve HSC-GT therapeutic effect.

P570

Mouse models of lysosomal storage diseases for a platform proof-of-concept study of LV-based HSPC-GT

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Pre-clinical and clinical data obtained in our Institute demonstrated the safety and efficacy of hematopoietic stem and progenitor cells-gene therapy (HSPC-GT) for some lysosomal storage disease (LSD) treatment. Upon transplantation, the circulating and tissue-resident progeny of *ex-vivo* transduced HSPCs releases a supraphysiological level of the functional enzyme that corrects non-hematopoietic cells more efficiently than standard enzyme replacement therapy and hematopoietic stem cell transplantation (HSCT). With the final aim to enlarge the application of HSPC-GT to other LSDs with skeletal involvement (Mucopolysaccharidosis IVA and IVB (MPSIVA and MPSIVB), Alpha-Mannosidosis (a-MANN)), we are currently characterizing the pathological skeletal, neurological, and immunological phenotype of *Galns-/-* (MPSIVA), *Glb1-/-* (MPSIVB), and *MAN2B1-/-* (a-MANN) mice. We have set a panel of skeletal analysis, including motor tests, full-body CT scan, histopathological evaluation of the knee joint, peripheral quantitative computed tomography (pQ-CT), and histomorphometry evaluation on eight males and eight females (16 mice) at 4 and 8 months of age, with wild-type age-matched littermate as controls. We will evaluate the neurological involvement in the case of neurological disease (MPSIVB, a-MANN) through a comprehensive assessment of cognitive, learning, memory, and social skills and histopathological evaluation of the brain cellular vacuolization and disorganization. Our laboratory generated *Galns-/-* mice using CRISPR/Cas9 technology in C57BL/6 mouse zygotes to delete the genomic region spanning from intron 1 to exon 2. We confirmed the absence of GALNS enzymatic activity in peripheral blood mononuclear cells (PBMNCs). We found reduced weight gain, shorter long bones at CT-scan, and weaker strength of upper limbs by grip test. At pQ-CT levels, *Galns-/-* mice presented reduced cortical area and stress index. We observed disorganization of the growth plate chondrocytes, which were swollen and engulfed compared to wild-type controls, signs of calcification, and inflammatory infiltrates in the articular cavity. The genetic deletion of our model causes the expression of a non-functional protein, which could be responsible for the exacerbated skeletal disease compared to previously published MPSIVA murine models. *Glb1-/-* mice were commercially available, bearing a 7-nucleotide deletion in the exon 5, which causes a premature stop codon. Glb1 enzyme was not functional in PBMNCs. Despite the absence of macroscopical skeletal abnormalities, *Glb1-/-* mice showed histopathological alterations in the joint synovia at 8 months of age. Preliminary neurocognitive tests demonstrated severe motor and cognitive alterations and abundant brain vacuolization. We will perform the same skeletal and neurological characterization on commercially available *MAN2B1-/-* mice. Our study will allow us to unveil the cellular and molecular characteristics of the selected disease models with the future aim of testing HSPCs-GT in these settings.

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AAV.GT5 gene therapy in a humanized mice model of OTC deficiency

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The liver is a preferred target for gene therapy against several genetic and metabolic disorders. Among many congenital metabolic diseases, ornithine transcarbamylase (OTC) deficiency has been a good candidate for liver-directed gene therapy. Since OTC is an essential enzyme in the hepatocytic urea cycle, a profound reduction of the OTC activity in the liver leads to a toxic level of ammonia in the blood. Some medications and a low-protein diet for a lifetime are effective but cannot prevent central nervous system sequelae. Invasive liver transplantation is the only curative treatment. Thus, the development of curative therapy is urgent. Previously, we generated a triple mutant AAV3 (AAV.GT5) vector with less reactivity to anti-AAV capsid neutralizing antibodies by introducing three substitutions (S472A, S587A, and N706A) on the surface loop of AAV3B capsid protein. Here we developed an AAV.GT5-based gene therapy in a mouse model of OTC deficiency. AAV.GT5-OTC vector expresses human OTC by the liver-specific promoter. Chimeric mice with humanized liver (PXB-OTCD) were generated by transplanting human primary hepatocytes obtained from the OTC deficiency patients into the spleens of 2 to 4-week-old, female, homozygous cDNA-uPA/SCID mice. The transduction efficiency of AAV.GT5 into human hepatocellular cell lines was similar to those of parental AAV3B, and it was 50-fold higher for hepatocytes derived from humanized PXB mice compared to AAV8 vectors which were commonly used for liver transduction. While most of the PXB-OTCD mice died within 4 weeks, AAV.GT5-OTC (1×10^{11} vg / mouse)-treated mice survived with a reduction of serum ammonia. In histology, the OTC enzyme was expressed in human-derived hepatocytes, and there were no abnormal findings, including liver fibrosis. After 12 weeks, AAV.GT5-OTC injection, levels of the OTC enzyme activity reached half of that of healthy hepatocytes in treated hepatocytes. It could be sufficient activity corresponding to that of carrier family members who do not have symptoms. For 12 weeks, no other toxicities of AAV injection was detected. Overall, AAV.GT5-OTC showed favorable efficacy and tolerability in a mice model of OTC deficiency. AAV.GT5-OTC appears to be a promising therapy for OTC deficiency.

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Assessing the efficacy and collateral effects of CRISPR-Cas13d-mediated *Hao1* RNA editing for Primary hyperoxaluria type 1: *in vitro* and *in vivo* evaluation

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Primary hyperoxaluria type 1 (PH1) is a rare metabolic disease characterized by oxalate overproduction in the liver and its toxic accumulation in the kidneys. Targeting the *Hao1* gene, which encodes glycolate oxidase (GO) protein, with CRISPR-Cas9 resulted in effective substrate reduction therapy in a preclinical model of PH1. However, the potential risk of permanent off-target modifications remains a safety concern. Here, we investigate the use of CRISPR-RfxCas13d, a programmable RNA editor that allows targeted gene knockdown without modifying the DNA. First, we chose the most effective gRNAs targeting *Hao1* mRNA in mammalian cells co-transfected with mouse *Hao1* mRNA and RfxCas13d fused to GFP. The tested guides (15) were able to reduce GO expression to undetectable levels in most cases. Collateral cleavage of exogenous non-targeted mRNAs was detected, as evidenced by a considerable drop in reporter protein (GFP) expression, whereas the expression of endogenous housekeeping protein remained unaffected. We assembled gRNAs with lower collateral effects in an array construction, together with the RfxCas13d gene under the transcriptional control of a liver-specific promoter in a recombinant AAV genome. Then, we evaluated their efficacy *in vitro*, revealing a substantial decrease in GO protein expression. These encouraging findings supported the *in vivo* evaluation of RfxCas13d. AAV8 vectors delivering RfxCas13d in combination with single or multiple gRNAs were designed and tested in mice. The animals were simultaneously injected with AAV8 carrying the luciferase reporter gene to test the potential collateral activity of RfxCas13d *in vivo*. The reduction in luciferase and RfxCas13d confirmed the collateral cleavage of exogenously expressed genes, which was substantially higher in animals treated with multiple gRNAs. However, GO protein levels were not significantly reduced, although the collateral effect suggested otherwise. A low cleavage efficacy or the activation of a compensatory mechanism to maintain GO levels might explain this finding. In summary, our *in vivo* data does not support the use of RfxCas13d targeting *Hao1* mRNA as an alternative substrate reduction therapy for the treatment of PH1.

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Development of AAV-mediated liver gene therapies for the stable correction of ornithine transcarbamylase deficiency

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Ornithine Transcarbamylase Deficiency (OTCD) is a rare genetic condition that impairs the urea cycle, a critical metabolic system that removes harmful ammonia from the body. The disease is caused by mutations in the X-linked OTC gene, resulting in a defective urea cycle, and ammonia and glutamine accumulation. Complete OTC deficiency manifests in the first days of life, with permanent neurological impairments and high mortality, while milder forms occur later in life with milder symptoms. Present treatments consist on dietary restrictions combined with ammonia scavengers, which are not curative, do not prevent hyperammonemia crisis, and do not provide patients with a good quality of life. The only curative option is liver transplantation, a very invasive procedure with many limitations.

Traditional AAV-mediated gene therapy has shown efficacy in treating adult patients with hemophilia, but still it poses substantial limitations in newborn and adolescent settings. The loss of viral DNA as the liver grows reduces the efficiency of AAV therapy and demands therapy re-administration, which is not possible due to the presence of anti-AAV neutralizing antibodies generated during the first vector administration. As a result, developing safer and more effective treatment options for OTCD in young patients is a medical need.

To address this need, our research team has employed a gene-targeting technique known as GeneRide, specifically targeting the albumin locus. By utilizing this approach, we have successfully demonstrated its effectiveness in a non-lethal animal model of OTCD. In our study, we also compared the outcomes of episomal and integrative liver gene therapy techniques in juvenile and neonatal mice, also in the presence of injury-induced hepatic regeneration. Dosing neonatal P2 Spf-Ash mice with an AAV donor vector carrying albumin homology arms and the hOTC cDNA, together with a second AAV expressing the SaCas9 and the sgRNA resulted in the long-term amelioration of the disease phenotype.

Episomal gene therapy in juvenile (P30) mice resulted in an important phenotypic improvement; however, the long-term therapeutic benefit faded in the case of liver regeneration. On the other hand, the integrative strategy demonstrated efficacy and long-term durability, while its therapeutic impact was not lost under hepatic regeneration conditions.

By establishing efficacy of the integrative gene therapy approach, we provide experimental support for an alternative and effective treatment for OTCD neonatal mice, laying the groundwork for more effective treatments for patients presenting pediatric and juvenile onset forms of OTCD.

P574

Murine Models of *PCCB* Deficiency Replicate Severe and Intermediate Phenotypes of Propionic Acidemia: Use to test AAV gene therapy

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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 nuclear encoded α- and six β-subunits with causative variants in either of the *PCCA* or *PCCB* genes found at equal frequencies in the patients. 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. While murine models and genomic therapies have been published for PA caused by PCCA deficiency, there are currently no publication describing the same for PCCB deficiency. To explore the pathophysiology of PA and generate a platform to test new therapies for PA, we have developed two new murine models of PCCB deficiency. CRISPR-Cas9 gene editing of the 14th exon of the murine *Pccb* gene yielded multiple alleles, including a 4 base pair deletion, designated *Pccb*$^{-/-}$, that results in a frameshift and premature stop codon in *Pccb* gene. *Pccb*$^{-/-}$ mice manifest a neonatal lethal phenotype and elevated plasma 2-methycitrate but had a significant increase in survival in comparison to untreated mutant controls (p<0.01) when treated with AAV9.CAG.PCCB at a dose of 1e11 vg/pup at birth. Due to the severe lethality displayed by the *Pccb*$^{-/-}$ mice, we designed a germline transgene to express the murine *Pccb* cDNA under the control of a muscle specific promoter (*Tg*$^{MCK\text{-}Pccb}$) to rescue the *Pccb*$^{-/-}$ mice. The resultant *Pccb*$^{-/-}$ *Tg*$^{MCK\text{-}Pccb}$ animals display increased survival, with most mice perishing at 1 month of age, moderate elevations of plasma methylcitrate in comparison to the *Pccb*$^{-/-}$ mice, and are growth retarded, very much like patients with PA. Next, we treated *Pccb*$^{-/-}$;*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 resulted in a significant increase in survival and growth in comparison to untreated *Pccb*$^{-/-}$ *Tg*$^{MCK\text{-}Pccb}$ controls . These new murine models replicate the clinical

and biochemical features of PA over a spectrum of severity, and can be used to test the effects of new genomic therapies for PCCB deficiency, such as systemic AAV gene therapy.

P575

An innovative platform approach for the pre-clinical development of ex-vivo gene therapy for lysosomal storage disorders with skeletal involvement

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Lysosomal storage diseases (LSDs) are characterized by the accumulation of undigested macromolecules causing severe multi-organ damage, which is only partially addressed by the currently approved therapies. Previous data obtained in our Institute proved the safety and efficacy of Hematopoietic Stem and Progenitor Cell-Gene Therapy (HSPC-GT) for some LSDs. Building on this experience and the common pathological mechanisms of LSDs, we are proposing an HSPC-GT approach to cure a group of rare/ultra-rare LSDs with skeletal involvement (Mucopolysaccharidosis IVA, Mucopolysaccharidosis IVB, Alpha-Mannosidosis) by using a standardized process in the framework of an innovative platform approach. We optimized Chemistry, Manufacturing and Controls, Non-clinical and Clinical development plans to generate a platform dataset complemented by specific disease data to generate a single combined Clinical Trial Application. To this aim, we generated 3rd-generation lentiviral vectors bearing the wild-type and the codon-optimized disease-specific transgene encoding for each enzyme (LV-GALNS, -GLB1, -MAN2B1) to transduce human HSPCs. Transduced cells showed proper clonogenic and proliferative capacity and significantly overexpressed the functional enzymes. We did not observe any advantages using the codon-optimized compared to wild-type transgene measured as protein expression and enzymatic activity. The myeloid progeny of transduced HSPCs efficiently released the enzyme in the cell supernatant, which restored the enzymatic activity in patients' derived fibroblasts. Osteoclasts derived from LV-GALNS and -GLB1 transduced cells abundantly secreted the proper enzyme, possibly serving as a resident source for skeletal cross-correction. We transplanted LV-GALNS and -GLB1 transduced HSPCs into sub-lethally irradiated NSG mice. Transduced cells engrafted and reconstituted the hematopoietic organs similarly to control cells. Importantly, we measured a significantly higher enzymatic activity in the bone marrow of mice transplanted with transduced cells (6x for GALNS; 1.2x for GLB1). To improve LV-GLB1 outcome, we optimized the GLB1 transgene (LV-eGLB1) and demonstrated enhanced expression in myeloid cells derived from LV-eGLB1 HSPCs, and in the bone marrow of NSG transplanted mice. Altogether, our pre-clinical data in vitro and in vivo support the platform development of HSPC-GT for LSD treatment.

P576

Intraocular and Systemic Gene Therapy for Gyrate Atrophy of the Choroid and Retina

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Gyrate atrophy of the choroid and retina (GACR) is due to ornithine aminotransferase (*OAT*) deficiency which causes hyperornithinaemia leading to retinal pigment epithelium (RPE) toxicity and impaired vision. Adeno-associated viral (AAV) vector-mediated *OAT* liver gene transfer lowers ornithinaemia in the *Oat*$^{-/-}$ mouse model of GACR and protects retinal structure and function but without full normalization. We investigated whether restoration of retinal OAT expression improves the retinal phenotype of *Oat*$^{-/-}$ mice. A single subretinal administration of an AAV vector encoding human *OAT* (AAV-*OAT*) results in intraocular OAT expression, reduced RPE abnormalities and improved thickness of the outer nuclear layer in *Oat*$^{-/-}$ mice up to 12 months of age. However, retinal electrical activity remained reduced, suggesting that hyperornithinaemia, which is not decreased by subretinal AAV delivery, impairs retinal function. We therefore combined systemic and intraocular administration of AAV-*OAT* in *Oat*$^{-/-}$ mice. Retinal electrical activity improved at 4 and 6 months of age compared to the two separate approaches. As expected, ornithinaemia lowered at both timepoints in mice administered with AAV-*OAT* systemically. This work shows the benefit of combined liver and retina gene supplementation for the treatment of GACR.

P577

Gene therapy for sialidosis

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Introduction
Patients with sialidosis (mucolipidosis type I) typically present with myoclonus, seizures, ataxia, cherry-red spots, and blindness because of mutations in the neuraminidase I (NEU1) gene. Currently, there is no treatment for sialidosis. In this study, we developed an adeno-associated virus (AAV)-mediated gene therapy for a Neu1 knockout (Neu1$^{-/-}$) mouse model of sialidosis.

Methods
The vector included the human NEU1 promoter, NEU1 cDNA, IRES, and human protective protein/cathepsin A (PPCA) cDNA. Viral vector was injected to neonatal mice (P2) via intracerebroventricular (ICV) injection or facial vein injection. Functional studies including rotarod, grip, and inverted screen were performed. Mice were sacrificed at 8 months old for immunohistochemistry evaluations.

Results
Untreated Neu1$^{-/-}$ mice showed astrogliosis and microglial activation in the brain and lysosomal storage in dorsal root ganglion (DRG) neurons, together with impaired motor function. Coexpression of NEU1 and PPCA in neurons and microglia by ICV or facial vein injection of neonatal Neu1$^{-/-}$ mice resulted in decreased astrocyte proliferation and microglial activation.

Rotarod performance and grasping power were both improved. In facial-vein-injected Neu1$^{-/-}$ mice, lysosomal storage in DRG neurons was decreased, and body weight was improved. There were no adverse events associated with the vector.

Conclusion
Neu1$^{-/-}$ mice showed astrogliosis and microglial activation in the brain, lysosomal storage in dorsal root ganglion (DRG) neurons, and impaired motor function. AAV-mediated gene therapy to coexpress NEU1 and PPCA improved these abnormalities without toxicity observed. Therefore, AAV-mediated gene therapy is safe and efficient for treating sialidosis.

P578

Overcoming current limitations in the development of RNA-based gene control systems

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Expression of the therapeutic protein in a gene therapy cassette can cause severe side effects. Off-target effects in the liver, kidneys and motor neurons in particular have been highlighted by the FDA as a major concern. One method for preventing these side effects is the use of genetic control systems, which form part of the gene therapy cassette and can be programmed to respond to internal or externally provided molecules to control gene expression in a defined manner. RNA-based control systems in particular have a number of advantages, including requiring no additional genetic components, their small size of <150 bp (freeing up space for the therapeutic payload) and their applicability across a broad range of therapeutics, most notably mRNA therapeutics where conventional DNA-based control systems (e.g. synthetic promoters) cannot be used.

The major bottleneck preventing the routine use of RNA-based gene control systems is their difficulty to engineer, with high time and resource costs and a limited guarantee of success. We analysed 25 RNA-based control systems that bind two proof-of-concept molecules, theophylline and tetracycline, representing the majority of those previously characterised. Many did not produce results consistent with those previously published in mammalian cells, showing either small fold changes or low specificity to the target molecules. We also demonstrated that many of the tools used for converting RNA-binding elements into functional control systems are not as modular as previously believed, being poorly adaptable to other target ligands.

To solve some of these key limitations in conventional development methods, which are largely trial-and-error in nature, we have developed a high-throughput contextual screening process and AI-guided improvement method. This screening methodology allows 10,000s (rather than 100s) of sequences to be screened in parallel directly in mammalian cells, and reduces costs >100-fold and lab time >10-fold compared to obtaining equivalent data using conventional methods. Meanwhile, we have developed an AI that can predict how well an RNA sequence will bind to a target ligand – a key aspect of designing RNA-based control systems – explaining >95 % of the variance in the data.

Removing the current limitations in control system design will allow their expansion from proof-of-concept molecules with limited therapeutic application to molecules with direct therapeutic relevance. This will enable the production of gene therapies that are tissue- and disease-state responsive, or can be controlled directly by clinicians after administration. Increased

control over these therapies will enhance patient safety and will be essential in the progression from the current generation of gene therapies that are limited to orphan diseases to larger indications.

P579

Development of an ex vivo precision gene engineered B cell medicine that produces highly active and sustained levels of acid sphingomyelinase for the treatment of Niemann-Pick Disease

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Niemann-Pick Disease (NPD) is an autosomal recessive lysosomal storage disease caused by acid sphingomyelinase (ASM) deficiency. Sphingomyelin accumulates in multiple organs leading to organ failure, and in the severest form causes death early in life. Currently, the only treatment for NPD is an enzyme replacement therapy (ERT) requiring an intensive dose escalation phase followed by Q2W maintenance intravenous infusions. Terminally differentiated human plasma cells derived from genetically engineered B cells (termed B Cell Medicines, BeCMs), offer natural longevity (persisting up to decades), capacity for high levels of protein secretion (up to 10,000 Ig molecules/cell/sec), and naturally allogeneic properties. This makes them an attractive platform for the sustained supply of biologics as compared to the ERT dose regimen required to achieve therapeutic benefit. We sought to harness the power of B cells by engineering them to produce therapeutic levels of ASM, thus offering a potential new treatment for NPD. In this study, primary human B cells were expanded, engineered by CRISPR/Cas9 genome editing with an AAV-delivered template for homology-directed repair (HDR) to insert a strong promoter followed by the ASM gene, *SMPD1*, into the CCR5 safe harbor locus, which showed up to 30% HDR. BeCMs are subsequently expanded and differentiated *in vitro*. Engineered BeCMs secreted ASM up to ~150 ng/1e6 cells/24hr as measured by ELISA. HAP1 *SMPD1* knockout cells exposed to supernatant from *SMPD1*-engineered BeCMs showed restored ASM activity. Liquid chromatography–mass spectrometry analysis demonstrated that HAP1 *SMPD1* knockout cells treated with supernatant from *SMPD1*-engineered BeCMs mitigated the accumulation of lyso-sphingomyelin and that BeCM produced ASM was approximately 10-fold more active than recombinant ASM made from CHO cells. Post-translational modification of BeCMs produced ASM as well as *in vivo* pharmacology in a NPD disease model are currently being investigated. In summary, we demonstrated the ability to transform the power of B cells into a platform for the advancement of B cell-based medicines. Successful production of ASM highlights the therapeutic modularity of our BeCM platform.

P580

Exploiting chimeric GALC enzymes with improved bioavailability to refine gene therapy approaches for globoid cell leukodystrophy

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Globoid Cell Leukodystrophy (GLD) is a severe neurodegenerative disease caused by deficient activity of b-galactosylceramidase (GALC). Current gene therapy (GT) strategies face the challenge of providing a sustained GALC supply in the central and peripheral nervous system (CNS, PNS). Leveraging previous results and supported by solid preliminary data, we aim to boost the therapeutic benefit of *ex vivo* lentiviral vector (LV)-mediated hematopoietic stem cell (HSC) GT in Twitcher (TWI) mice (GLD model) by coupling neonatal treatment, safe enzyme overexpression in effector cells, and enhanced bioavailability/metabolic correction of enzyme-deficient cells provided by chimeric GALC enzymes.

Early treatment is paramount in the severe TWI model since storage accumulation and neuroinflammation start in the first week of life and lead to rapid degeneration and premature death (average lifespan: 39 days). To this end, TWI HSCs were transduced with LVs expressing the chimeric or wild type (WT) version of GALC and transplanted (TX) in TWI newborn mice after sub-lethal myeloablation (busulfan). Mice transplanted with WT total bone marrow (tBM) or WT HSCs were used as controls. We highlighted a low chimerism of WT HSCs (10-15%) as compared to tBM (50-60%) in the peripheral blood (PB) of TX mice. This was associated with low engraftment of donor-derived myeloid progeny in the brain and a modest survival advantage of HSC-TX mice (average lifespan: 43 days) with respect to the tBM-TX counterpart (average lifespan: 49 days). We achieved safe and robust GALC overexpression (up to 14x the physiological levels) in donor TWI HSCs transduced with LVs expressing the chimeric GALC. Still, the PB chimerism in TWI mice transplanted with LV-transduced HSCs was comparable to that of WT HSCs, likely explaining the absence of a clear survival advantage associated with GALC overexpression in donor cells or to the chimeric vs. WT enzyme.

These results suggest that the suboptimal conditioning/low level of donor cell engraftment and the use of a severe animal model hamper the assessment of treatment-related therapeutic efficacy. To overcome these limits, we will take advantage of a conditional GLD mouse in which the early post-natal *galc* deletion results in slower disease progression and prolonged survival (>90 days). We expect that HSC GT performed in 1-month-old GLD conditional mice after lethal myeloablation would result in high PB and BM chimerism as well as robust engraftment of donor-derived myeloid progeny in the CNS/PNS. The use of these novel experimental settings will provide proof-of-principle of the feasibility and safety of HSC GT for GLD and the potential advantage of chimeric vs. WT GALC in enhancing enzyme bioavailability to increase the survival and rescue CNS/PNS pathology of treated GLD mice.

P581

Redesigning Pompe Disease gene therapy tools to promote paracrine amelioration

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Pompe Disease (OMIM: 232300) is a fatal neuromotor disorder characterized by the progressive accumulation of glycogen in skeletal muscle, liver, and heart. The disease is caused by inherited mutations in the human GAA gene, which reduce the activity of the lysosomal acid alpha glucosidase enzyme (GAA). This leads to the buildup of lysosomal glycogen deposits, resulting in impaired cellular structure and severe muscle tone problems. Currently, there is no known cure for Pompe Disease. The only approved treatment is enzyme replacement therapy (ERT), which temporarily slows down the loss of muscle function but does not halt the progression of the disease.

Given the success of gene therapy in other lysosomal storage disorders, our objective was to develop improved gene therapy tools specifically for Pompe Disease, it has been previously described that the natural GAA peptide is not optimal for cross-correction strategies. Our bioinformatic analyses revealed that the GAA peptide has suboptimal signaling capabilities, limiting its secretion. To address this, we reevaluated the GAA pro-peptide and made modifications to its leader peptide and non-catalytic N-terminal domains. Our aim was to enhance local paracrine distribution and promote uptake as well as CI-M6P-independent interactions with target tissue cells. Our designs retained the C-terminal GAA lysosomal maturation and active enzymatic domains. Next, we redesigned our constructs considering codon choice and vectorization. For initial screening purposes, we substituted the GAA active peptide (76 kDa) with the eGFP peptide (30 kDa). By doing so, we created paired therapeutic and reporter constructs that shared identical trafficking, secretion, capture, and maturation peptides. We successfully produced and verified the reporter vectors to evaluate and rank our designs, selecting the most promising candidates for preclinical evaluation in lab-generated Pompe Disease models.

These findings lay the foundation for translational studies aimed at identifying potential gene therapy candidates to improve the clinical outcomes of Pompe patients.

P582

Next generation, allogeneic cell therapies for Type 1 Diabetes – utilising Gene Editing induced Gene Silencing (GEiGS) technology to address current challenges

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Development of cell therapies as a treatment for Type 1 Diabetes (T1D) has shown considerable progress, but there remain a number of key hurdles and challenges to allow wider adoption. The majority of current approaches use engineered cells that are delivered to immune-suppressed patients and are encapsulated in devices which restrict vascularisation and full integration with the recipient.

We have identified key areas where our core Gene Editing induced Gene Silencing (GEiGS) technology can directly help address these challenges, facilitating development of the next generation of cell therapies for T1D with improved efficacy and safety profiles. GEiGS is a new gene silencing approach that recodes endogenous miRNAs to redirect them towards new targets, taking advantage of cell type-specific miRNA expression patterns to control gene expression in a stable, tunable and programmable manner.

Here we present data on use of GEiGS in generation of hypoimmune pancreatic cells via modulation of components of the MHC-I complex. In this context, GEiGS provides a number of unique features; 1. GEiGS elicits highly tunable gene silencing via minimal genome edits; 2. silencing is linked to cell identity, reducing the tumourigenic risk from contaminating immune-privileged immature cells (iPSC or progenitors cells), or from de-differentiation of mature therapeutic cells; 3. as GEiGS does not require transgene insertion, the risk of positional or epigenetic silencing and variegated expression of transgenic tolerising factors is mitigated, resulting in differentiation of homogenously hypoimmune pancreatic cells.

P583

Exploiting cancer cell metabolism through a multiple metabolic hit approach

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Identifying targetable differences in the metabolism between primary and cancer cells that can be exploited for therapeutic purposes holds immense promise in the field of cancer. Cancer metabolism is associated with aerobic glycolysis and limited uptake of pyruvate. One difference causing this distinct metabolic profile is the differential expression of the *Mitochondrial Pyruvate Carrier 1* (*MPC1*) gene. *MPC1* has been described to be downregulated in various cancer types leading to poor survival rates in patients. In this study, we aim to reverse the effects of the downregulation by overexpressing the *MPC1* gene using modified RNA (modRNA). After treatment of different cell lines and human organoids with the *MPC1* modRNA, the proliferation of the cells as well as of the organoids was slowed down. The overexpression of *MPC1* was combined with the inhibition of the Stearoyl-CoA Desaturase 1 (SCD1) enzyme, which is

responsible for converting saturated fatty acids into unsaturated ones. The inhibition of the enzyme was proven to be toxic for the cells, by causing saturated fatty acids to accumulate inside them. To further enhance the effects of this approach and decrease the viability of the cells, the SCD1 inhibition was combined with the treatment of cells with fatty acids. Overall, this multiple metabolic hit strategy we propose could be implemented as a cancer-specific type of therapy targeted exclusively towards the intrinsic metabolism of cancer cells.

P584

Wharton's Jelly MSC Exosomes Loaded with Apelin: A Promising Therapy for Insulin Resistance Reversal

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Diabetes, predominantly characterized by β-cell dysfunction and insulin resistance, remains an intricate medical concern. While the peptide Apelin holds potential in alleviating insulin resistance, its utilization has been curtailed due to suboptimal targeting and restricted half-life. To circumvent these constraints, we employed an innovative approach, utilizing Apelin-laden exosomes derived from Wharton's Jelly-derived mesenchymal stem cells (WJMSCs). This novel strategy could potentially revolutionize the treatment of insulin resistance in diabetes, effectively circumventing previously identified shortcomings.

We used a high-fat diet and a low-dose streptozotocin (STZ) injection-induced type 2 diabetes mouse model in our study. Following the identification and isolation of human WJMSCs, we introduced apelin-expressing lentiviral particles. This led to the creation of Apelin-expressing WJMSCs, as confirmed via flow cytometry, western blotting, and qPCR analysis. We isolated and exhaustively characterized exosomes from these modified cells, termed Apelin-WJMSCs-exos.

For in vivo analysis, type 2 diabetic mice received an injection of either 1×10^10 particles of Apelin-WJMSCs-exos or a comparative dose of WJMSCs-exos, via the tail vein, seven days post-STZ administration. We monitored plasma glucose levels and conducted glucose and insulin tolerance tests (OGTT and IPITT respectively). Confocal microscopy and immunocytochemical analysis were utilized to assess the therapeutic efficacy of each group and quantify islet beta cells. Simultaneously, we analyzed GLUT4 expression in 3T3-L1 adipocytes and C2C12 skeletal muscle insulin resistance models, and probed for changes in AKT, eNOS, and AMPK pathways.

Administration of Apelin-WJMSCs-exos resulted in a significant decrease in blood glucose levels in type 2 diabetic mice, along with increased proliferation of endogenous pancreatic β-cells. Additionally, sustained administration led to increased plasma levels of insulin and C-peptide. Our study revealed that Apelin-WJMSCs-exos influenced the activity of AKT, eNOS, and AMPK in skeletal muscle, adipose tissue, and cells of mice through phosphorylation.

Our findings underscore the therapeutic potential of Apelin-loaded exosomes derived from WJMSCs for the alleviation of insulin resistance in type 2 diabetic mice. The data indicates superior efficacy of these exosomes compared to those exclusively loaded with WJMSCs, suggesting a promising prospect for a new, clinically viable treatment approach for type 2 diabetes.

P585

Therapeutic efficacy of VTX-801 Wilson's disease mice at an advanced stage the disease

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Wilson disease (WD) is a disorder of copper metabolism that can present with hepatic, neurologic, or psychiatric disturbances, or a combination of these. If untreated WD is a life-threatening condition.

Recently, we have demonstrated that an adeno-associated vector (AAV) serotype Anc80 carrying a reduced version of the human ATP7B cDNA (VTX-801) provides long-term correction of copper metabolism in WD young male and female WD mice.[1] Here we challenge the therapeutic efficacy of the VTX-801 treating animals with a more advanced disease. WD mice of 12, 16 and 20 weeks of age showed clear histological abnormalities, with a significant inflammatory infiltrate, hepatocyte hypertrophy and necrosis.

12-, 16- and 20-weeks-old WD mice received a dose of VTX-801 identified as therapeutic in young animals. The therapeutic effectiveness was evaluated 6 months after the vector administration. Twenty-four-hour urine samples were collected to measure urinary copper excretion and blood was extracted for the evaluation of biochemical and copper-related parameters. Mice were sacrificed and livers, kidneys and brains obtained. The copper concentration was determined in all organs and with the liver a more extended analysis was performed: Liver histology was evaluated; Sirius red staining was performed for the evaluation of fibrosis and IHC was performed to assess bile duct proliferation and immune cell infiltration. In addition, vector genome copies were also determined.

Based on the results obtained so far, we can conclude that VTX-801 treatment had significant beneficial effects at all ages. However, lower therapeutic efficacy was observed when vector administration was performed in the more advanced stages of the disease (20 weeks). Some disease biomarkers such as serum liver transaminases and bile acids levels and some features of liver histology including liver fibrosis and histology significantly improved after treatment but were not completely corrected. A lower transduction efficiency associated with disease progression was observed and might explain a reduced therapeutic effect.

Our data demonstrate that VTX-801 administered to animals showing significant liver histological alteration and a sustained liver damage was able to prevent the progression of Wilson's disease and improve most of the pathological features of the disease indicating that gene therapy is safe and efficient even at advanced stage of the disease.

P586

High-efficiency genetic correction of primary hyperoxaluria type 1 causing *AGXT* variant, c.508G>A (Gly170Arg) with an adenine base editor in patient-derived fibroblasts

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Primary hyperoxalurias are autosomal recessive diseases that are associated with oxalate overproduction, nonsoluble calcium oxalate accumulation in various tissues, especially in the kidney, and resulting renal failure. The most common type of hyperoxaluria, primary hyperoxaluria type 1 (HP1), results from pathogenic mutations in the *AGXT* gene encoding liver-specific peroxisomal alanine-glyoxylate aminotransferase. HP1 has an estimated incidence rate of 1 per 120,000 live births in Europe. The most common pathogenic *AGXT* mutation, c.508G>A (Gly170Arg), occurs in 24 to 37 % of all HP1 patients and is associated with significant catalytic activity. Adenine base editors (ABE) are precision genome editing tools that can correct C·G>T·A transition mutations in a narrow, predetermined genomic locus with high efficiency, but without introducing double-stranded DNA breaks. We proceeded to test the potential of ABE to correct this pathogenic variant in an *in vitro* setting.

We cultured fibroblasts from a skin biopsy of a patient carrying compound heterozygous mutations (*AGXT*, c.508G>A (Gly170Arg), c.673_676delAAGG). In a single electroporation reaction, we corrected the pathogenic missense variant using *in vitro* transcribed ABE mRNA and single guide RNA, and simultaneously reprogrammed the patient fibroblasts into human induced pluripotent stem cells (hiPSC) using plasmids expressing the classic pluripotency factors. In this reaction, we reached 92 % on-target editing, without significant proximal bystander editing. To model the disease *in vitro*, we differentiated genetically corrected monoclonal hiPSC lines and their non-corrected isogenic counterparts into hepatocyte-like cells using established differentiation protocols.

Considering the devastating phenotype of primary hyperoxaluria type 1, and the high-efficiency editing reached *in vitro*, we suggest that the genetic correction of the *AGXT* missense variant could be further studied and developed into novel therapies.

P587

Dissecting the mechanisms of myeloid-to-neural enzymatic cross-correction in the context of hematopoietic stem cell gene therapy for Metachromatic Leukodystrophy

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Metachromatic Leukodystrophy (MLD) is an autosomal recessive disease caused by defects in Arylsulfatase A (ARSA), a lysosomal enzyme that degrades sulfatides. Neurological manifestations are related to white matter loss in the central and peripheral nervous systems (CNS, PNS) associated with 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 provides superior benefit to MLD patients as compared to conventional allogeneic HSC transplantation (HSCT). While the key role of metabolically-competent HSC myeloid progeny in providing immunomodulation and neuroprotection is recognized, the exact mechanism of myeloid-mediated enzymatic cross-correction of MLD neural cells is debated.

Here, we showed that LV.ARSA-transduced MLD human monocyte-derived macrophages actively released a functional ARSA enzyme that proficiently cross-corrected ARSA-deficient neurons and glial cells derived from MLD patient-specific induced pluripotent stem cells (iPSCs). ARSA over-expression did not impact the M1/M2 macrophage polarization that, in turn, did not influence ARSA secretion from myeloid cells, as shown by the comparable ARSA uptake in MLD hiPSC-derived neurons and glia through mechanisms that are partly mediated by the mannose-6-phosphate receptor. Indeed, our data in human myeloid cell lines suggest that the transgenic ARSA enzyme is post-transcriptionally modified through the phosphorylation of mannose-6 residues.

Taking advantage of unique *in vitro* human disease models, we demonstrated the occurrence of a consistent myeloid-mediated enzymatic correction of ARSA-deficient neurons and glial cells in a clinically-relevant HSC GT setting.

P588

Survival analysis of propionic acidemia patients with liver transplant

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Propionic acidemia (PA) is a serious metabolic disorder characterized by impaired metabolism of certain amino acids. Liver transplantation is a treatment option which leads to improvements in disease symptoms and progression. However, the available evidence on the impact of transplantation on long-term survival is scarce. A systematic literature review (SLR) and survival analysis were conducted to address this gap by collecting individual patient data. The SLR was performed in February 2023 across relevant scientific databases without publication date limit. A Kaplan-Meier curve was created using the age at death or loss of follow-up data of the identified 94 transplanted PA patients. 17 deaths were reported between the age of 3 months and 19 years and the oldest patient observed alive post-transplant was 33 years old. At age 33, the survival probability was calculated at 62% among PA patients. As compared to the general population of 98% at the same age, this probability is much lower. Disease-specific mortality rates were calculated for the following age bands: 0-1, 1-5, 5-10 and 10-33 years. By extrapolating mortality rates from the 10-33 years age band to lifetime time horizon, the median survival of PA patients post-transplant was estimated to be 40 years with a 95% confidence interval of 12-70 years. The results of the current SLR and survival analysis highlight a substantial survival deficit of PA patients despite liver transplantation. The high unmet medical need in the disease area calls for the development of new innovative therapeutic approaches.

P589

AAV-mediated gene therapy shows sustained high level expression of blood coagulation factor VII *in vivo*

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FVII deficiency is a rare autosomal recessive bleeding disorder resulting from variants in the gene encoding FVII with a prevalence of approximately 1:300.000-500.000 individuals. The only available treatment is a recurrent and lifelong factor concentrate infusion, which is both a burden for the patients and is linked to high costs for the healthcare system. Aim of the project is to investigate whether AAV-directed gene transfer approach for FVII deficiency can achieve durable and functional expression of human FVII (hFVII) in vivo using wild-type viral capsid, as well as newly engineered liver-specific capsids.

We developed an AAV-based gene therapy platform designed to direct high level expression of FVII into hepatocytes. We performed capsid engineering to further improve targeting of AAV to hepatocytes. We also developed a collection of rationally designed liver-specific promoters and evaluated multiple codon optimization strategies.

As a first step, we compared the efficacy of AAV5 and AAV8 to direct expression of FVII. AAV8 appeared to be significantly more efficient in directing expression of FVII when compared to AAV5. Analysis of tissue-distribution of AAV5 and AAV8 revealed that both lung and liver was targeted by AAV5 whereas AAV8 preferentially targeted the liver. Our proof-of-concept study in wild-type mice showed that AAV8-directed gene therapy could successfully express and release biologically active hFVII protein into the circulation. At a dose of 2E10 GC/animal, we observed that different cDNA sequences can greatly impact the gene therapy outcome. Supraphysiological FVII levels up to 20 U/mL were obtained when a FVII transcript with a 22 amino acid deletion in

the propeptide (hFVII-22) was used whereas wild-type FVII was expressed at levels of 3 U/ml. Long-term high level expression of FVII for up to 48 weeks post-injection was observed using a codon-optimized version of hFVII-22 that was expressed under the control of a liver-specific promoter. To further improve liver targeting of AAV8, we employed rational capsid engineering of surface-exposed loops that resulted in several capsid-engineered AAV variants. In vitro results showed that these capsid variants infected liver HepG2 cells significantly better than their parental unmodified version.

Our results showed that we have established a novel liver-specific gene therapy platform that can successfully be used for sustained expression of FVII in vivo. The results obtained support further pre-clinical development of FVII gene therapy. The AAV based gene therapy platform we have developed may also be used for development of novel gene therapy based approaches for other liver-specific disorders.

P590

Molecular design of high-capacity adenoviral vectors as large transgene carriers exemplified by von Willebrand factor

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Von Willebrand Disease (VWD) is a genetic bleeding disorder and comes with symptoms like menorrhagia or extended blood loss after injuries. It is caused by mutations in the gene encoding von Willebrand factor (VWF), which is a 540 to 20000 kDa glue-like plasma protein dependent on the formation of multimers. It has the function of stabilizing and carrying coagulation-factor VIII and to recruit platelets at sites of vascular injury to help cessation of bleeding. High efficient VWF-gene delivery and lifelong gene expression are the main goal of the gene therapy setting. However, due to the large transgene size for VWF (8.4 kilobases (kb) without promotor elements), predominantly used vector systems based on Adeno Associated Virus or Lentivirus are not of choice. To deliver these large transgenes, we applied high-capacity adenoviral vectors (HCAdVs) with transgene carrying capacities of up to 36 kb. To achieve lifelong treatment, we established a hybrid-vector system that enables transgene delivery and its subsequent integration into the host genome using Sleeping Beauty (SB) transposon system that consist of transgene as transposon and the hyperactive SB100x transposase.

Initially, we generated a novel HCAdV (HCAdV-VWF-GFP) encoding VWF under the control of a liver-specific promoter HCRHPi flanked by SB transposon inverted repeats and Flpe recombinase recognition sites enabling excision of the SB transposon VWF expression cassette from the HCAdV genome. In a mouse model for severe VWD, a single intravenous administration of HCAdV-VWF-GFP co-delivery with HCAdV-SB100x resulted in high and stable VWF levels up to 2 years after gene transfer. However, liver-expressed VWF showed incomplete multimerization, impairing the hemostatic activity of liver-expressed VWF. To improve multimerization, expression in endothelial cells, the natural site of VWF synthesis, is needed. Therefore, HCAdVs with improved VWF transgene expression cassette needed to be generated in which VWF expression is controlled by of an endothelial cell specific promotor.

To achieve a flexible transgene design and to insert a broad size range of transgenes, we designed a modular cloning set up for HCAdV genomes for flexibility during cloning for different transgene sizes. We generated several HCAdV genomes, which contain different numbers of a 4.2 kb non-coding DNA fragment. For HCAdV-production, a genome size in the range of 28-37 kb is necessary to maintain the genome stability and efficient vector packaging. Therefore we constructed a plasmid tool box with one to four noncoding DNA fragments, allowing efficient insertion of transgenes in the range of 3 kb to 28 kb. Development of the modular system for HCAdV-production offers easy and efficient cloning possibilities for a wide range of gene therapeutic applications. Using our novel modular system, we are currently constructing the HCAdV contains the SB transposon with VWF under the control of endothelial-specific promoter (huTie2). This new vector system can then be combined with the SB100x transposase for somatic integration, endothelial-specific VWF expression with proper multimerization to finally achieve therapeutic function.

P591

Adenovirus vectors lacking thrombosis-associated PF4 binding show improved safety profile and applicability as vaccine or gene therapy candidate vectors

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The adenoviral (Ad) vector based AstraZeneca and Janssen COVID vaccines have been associated with rare cases of thrombotic thrombocytopenia syndrome (TTS, also called VITT), a condition which depends on Ad binding to the blood protein Platelet Factor 4 (PF4).

Harnessing the broad diversity of natural and synthetic Ads, we strived to identify Ad types lacking PF4 binding; establish platforms for gene therapy, oncolytic and vaccine vector development using these vectors; locate the binding site(s) of PF4 on the capsid of Ad type 5 (Ad5); and decipher the impact of PF4 binding on Ad tropism and immunogenicity.

The PF4 binding ability of 38 natural Ad types and a collection of genetically or chemically modified Ad5 hexon or fiber variants was assessed using independent techniques including SPR and ELISA-qPCR. Unlike most tested vectors, Ad11, Ad13, Ad34 and Ad80 did not bind to PF4. Likewise, the deletion or shielding through PEGylation of the hexon hyper-variable region (HVR) 1 of Ad5 ablated its PF4 binding. PF4 preferential binding on Ad5 HVR1 loop was confirmed by cryo-EM. An Ad5 variant carrying the HVR1 loop of Ad34 displayed decreased but still detectable PF4 binding, possibly due to its similar steric structure compared with wild-type Ad5.

Interactions with PF4 substantially modified Ad binding to or transduction of numerous immortalized or primary cell types, suggesting that PF4 may influence the tropism of vaccines and other clinical Ad vectors in undesired ways. These effects were not mediated by Ad receptors or glycocalyx but can be enhanced or inhibited by other serum proteins including coagulation factor X.

In order to study the immunogenicity of Ad-PF4 complexes, we isolated human peripheral neutrophils and differentiated macrophages from several healthy volunteers. Although Ad-PF4 interactions did not significantly influence NETosis and Th1 cytokines expression levels in single-population cultures, they increased Ad intermalization in presence of thrombocytes. This phenomenon may contribute to amplify TTS-related immune response and pathogenesis.

Finally, we explored the potential of the Ad types lacking PF4 binding as candidate therapeutic vectors. Notably, Ad34-derived vectors displayed efficient transduction of several hematopoietic populations including stem cells. In addition, the safety and immunogenicity of PF4-negative preclinical vaccine vectors expressing a SARS-CoV-2 spike transgene is currently investigated in mice.

We hope that these findings open the way for the development of safer vaccine or gene therapy vectors with decreased or suppressed risk of TTS.

P592

Proinflammatory preconditioning of human menstrual blood-derived mesenchymal stromal cells (MenSCs) enhanced the release of miRNAs associated with cisplatin chemosensitivity in ovarian cancer.

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Ovarian cancer is a major cause of mortality among women globally, so there is a need to explore innovative therapeutic approaches to improve treatment outcomes. Cisplatin (DDP), a platinum-based chemotherapeutic agent, has been extensively used in ovarian cancer treatment. However, the development of drug resistance and the associated adverse effects generate the need to develop combination therapy. In this context, the combined treatment with human menstrual blood-derived mesenchymal stromal cells (MenSCs), a non-invasive and readily available source of mesenchymal stem cells with regenerative and immunomodulatory properties, could become a future approach as adjuvant therapy. Moreover, MenSCs have shown promising potential in cancer therapy due to their ability to migrate to tumor sites and modulate the tumor microenvironment. MenSCs exert these actions in a paracrine manner through their secretomes, which enclosure multiple miRNAs, among other components, that can modulate the expression of genes involved in the cellular response to drugs. Indeed, cell preconditioning strategies modify the miRNAs expression pattern which could increase the effectiveness of these treatments. Therefore, the aim of this work is to evaluate the effect of proinflammatory preconditioning with IFNγ and TNFα on miRNAs released by MenSCs and its possible implication in the therapeutic efficacy of DDP in the treatment of ovarian cancer. Following transcriptomic analysis of miRNA released under basal and proinflammatory conditions, out of 19 differentially expressed miRNAs (*p*-value>0.05 and FDR>0.01), 6 are involved in modulating the response to cisplatin in ovarian cancer. hsa-miR-199a-3p, hsa-miR-34a-5p, hsa-miR-27a-3p, hsa-miR-146b-5p, and hsa-miR-155-5p enhance sensitivity to cisplatin while hsa-miR-21-3p enhances chemoresistance. In conclusion, proinflammatory priming of MenSCs appears to induce the release of miRNAs that increase sensitivity to DDP. Thus, the miRNAs from secretome in combination with cisplatin offer

a novel approach that could improve the efficacy of ovarian cancer treatment, though overcoming chemoresistance.

P600

Isogenic human cell models to unravel the underlying mechanism of cystinosis myopathy

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Cystinosis is a rare, autosomal recessive disease caused by lack or dysfunction of cystinosin (CTNS) leading to lysosomal cystine accumulation. The disease is systemic, but first primarily affects the kidneys. Renal transplantation, cysteamine treatment and improved medical care improved survival and the quality of life, which in turn led to the emergence of additional systemic phenotypes like myopathy. Muscle weakness forms a major concern leading to life-threatening events in patients, like swallowing difficulties and respiratory insufficiency. The etiology of cystinosis myopathy remains to be elucidated. We engineered human muscle cell-based models to better understand the pathophysiology of cystinosis myopathy with the potential to develop new therapies. Using CRISPR technology, we generated polyclonal, isogenic human *CTNS* knock-out (KO) myoblasts, corroborated at gDNA level and by elevated cystine levels in metabolomic analysis. Growth characteristics were not altered upon CTNS depletion and no other metabolites were affected. *CTNS* cDNA complementation using lentiviral vectors reverted the cystine accumulation to wild-type (WT) levels. Next, differentiation of the myoblasts into myotubes was assessed, showing no significant difference in the fusion index for *CTNS* KO compared to WT myoblasts. Further, we assessed several key regulators of myogenic differentiation. We observed decreased protein expression for at least two of these proteins in *CTNS* KO cells compared to WT cells. Interestingly, *CTNS* cDNA addition rescued these phenotypes. As a next step, we will investigate pathways involved in myogenic differentiation by performing proteomic analysis and RNAseq analysis.

P601

AAV6-cTnT-Hcn2/SkM1 gene transfer enables a transient biological pacemaker activity in a porcine model of CAVB

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Electronic pacemakers have successfully been used for decades in the treatment of bradycardia. Despite their therapeutic efficacy, they retain important shortcomings inherent to their

hardware-based design that are difficult to resolve. In an effort to develop hardware-free pacemakers, we have engaged into the designing and testing of gene therapy-based biological pacemakers by means of ion channel overexpression. Combined adenoviral gene transfer of the pacemaker channel HCN2 and the skeletal muscle sodium channel SkM1 has previously been demonstrated to deliver highly efficient short-term biological pacing in dogs with complete heart block. In the present study, we report our progress with regard to Hcn2/SkM1 gene delivery mediated by adeno-associated virus (AAV), to generate long-term biological pacing in a porcine model of complete atrioventricular block (CAVB). Functional biological pacemaker studies were conducted in a porcine model of radiofrequency ablation-induced CAVB. Four weeks after the ablation, animals were distributed in four different groups: non-injected, saline, and AAV6-Hcn2/SkM1, with or without immunosuppression. All animals were followed for another four to six weeks to evaluate in vivo biological pacemaker performance and then hearts were harvested to assess transduction efficiency. One week after gene transfer, Hcn2/SkM1-transduced animals displayed a notable increase in maximal beating rates above 100 beats/min, mean heart rates only modestly trended up, yet a remarkable reduction of electronically paced beats was evident with a backup pacing of less than 60%. Quantification of gene transfer efficiency in the injection sites revealed remaining expression levels of both transgenes in transduced animals at four- and six-weeks post-injection. In conclusion, AAV6-mediated Hcn2/SkM1 gene transfer in CAVB pigs resulted in transient biological pacemaker activity in the first week after injection, although transgene expression levels were detectable four to six weeks post-transduction.

P602

Highly Efficient and Durable AAV-mediated Skipping of DMD Exon 53 in hDMDdel52/mdx Mice Results in Sustained Therapeutic Levels of Dystrophin

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Duchenne Muscular Dystrophy (DMD) is caused by mutations in the DMD gene, encoding Dystrophin. Of these mutations, roughly 80% are amenable to exon skipping, a therapeutic approach in which specific exon(s) are excluded from the mature mRNA to restore the open reading frame disrupted by mutations. The result is a nearly full length and functional dystrophin protein. We have developed, a self-complementary (sc) AAV vector expressing three antisense sequences on a modified U7 small-nuclear RNA (snRNA) scaffold that achieves efficient skipping of dystrophin exon 53 in multiple cell lines and in vivo in the hDMDdel52/mdx mouse model.

Multiple anti-sense sequences were chosen to target exonic splicing enhancer sequences in intron 52 and exon 53, and the splice acceptor site in intron 52, and linked to a U7 snRNA scaffold. Constructs were evaluated in cultured human rhabdomyosarcoma (RD) cells and assayed by digital droplet PCR for skipping efficiency. The lead construct (AAV.3XU7.Ex53.3AS) was evaluated in immortalized human myoblasts derived from a patient with a deletion of DMD exon 52 and found to promote up to 80% skipping of exon 53 and robust expression of dystrophin.

Next, we performed in vivo proof-of-concept studies in hDMDdel52/mdx mice, which express a human dystrophin mRNA without exon 52 and are amenable to exon 53-skipping therapies. Three months post AAV infusion, skipping

efficiency of exon 53 in heart was 87% (low-dose) and 96% (high-dose), which corresponded to 35% and 50% of wildtype (C57Bl/6J) levels of dystrophin protein, respectively.
In the gastrocnemius, 53% (low-dose) and 84% (high-dose) skipping resulted in 19% and 37% of w ildtype protein. Histological analysis of gastrocnemius muscle revealed stable restoration of dystrophin at the sarcolemma along with improvement in muscle pathology when analyzed by Sirius red and hematoxylin-eosin staining. As early as one-month post vector treatment, significant reduction in fibrosis and muscle regeneration were observed at either dose compared to mice treated with a scramble U7 snRNA vector. A direct comparison of quadriceps muscles at 1- and 3-months after vector administration revealed a 50% (high-dose) increase in dystrophin protein over the period. We are currently evaluating this vector in a long-term (12-month) study. Preliminary data from an ongoing functional evaluation study suggest improvement in muscle function by grip strength and limb hanging tests after treatment with the AAV.3XU7.Ex53.3AS vector. The results from a pilot NHP study demonstrated up to 90% skipping in the heart and up to 30% skipping in Gastrocnemius muscle by ddPCR providing evidence for efficiency of the construct in a large animal model. Additionally, we have developed a vector for skipping exon 51 of dystrophin mRNA, AAV. 3XU7.Ex51, which provided similar results in patient myoblasts in terms of skipping efficiency and dystrophin restoration and in vivo evaluation of this vector in hDMDdel52/mdx mice is ongoing. Altogether, our results provide compelling proof-of-concept data supporting the development of vectorized exon-skipping therapies for the treatment of DMD.

P603

Targeted genetic Intervention with Cas9 and a mutation-specific single guide RNA to rescue survival and cardiac deficits in 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 and currently lacks an effective treatment. This exceptionally rare condition is characterized by muscle weakness, hypotonia, joint contractures, respiratory insufficiency, spinal rigidity, and cardiac anomalies, which can result in sudden death. Our primary objective is to develop efficient gene therapies for L-CMD. In this study, we investigated the potential of CRISPR/Cas technology in treating this disease using mutation-specific guides. First, we demonstrated the specificity of this approach for targeting and eliminating the mutant allele while minimizing the impact on the wild-type allele, utilizing various mouse and human cell models. Subsequently, we employed AAV9 vectors as delivery vehicles to evaluate the efficacy of this gene therapy strategy in mice harboring either one or two copies of the Lmna c.745C>T, p.R249W mutation. These mice exhibited either a late onset cardiomyopathy phenotype or an early onset metabolic phenotype, respectively. Remarkably, a single preventive dose of AAV9 particles significantly increased the median survival of homozygous Lmna-R249W/R249W mice in the metabolic scenario, even at low levels of CRISPR-mediated editing in the target tissues. Likewise, heterozygous mice, which manifested a cardiomyopathy phenotype resembling that of

L-CMD patients, positively responded to the gene therapy involving AAV9-mediated delivery of Cas9 and sgRNA specifically targeting the c.745C>T mutation. In this cardiac context, survival was significantly prolonged, accompanied by the rescue of cardiac abnormalities. Our results provide the initial evidence for the potential of CRISPR-based therapy as a promising treatment approach for L-CMD.

P604

AAV-mediated RBM20 augmentation prevents dilated cardiomyopathy caused by RBM20 RS-domain mutations

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The cardiac splicing factor RNA binding motif 20 (RBM20) regulates splicing events of several targets regulating sarcomeric structure (for eg:Titin) and calcium handling (for eg: RyR2) in the myocardium.

Missense mutations of RBM20 have been associated with genetic forms of dilated cardiomyopathy
(DCM). Although mutations are found throughout the gene, there is a hotspot found within the RS domain. Specifically, RS domain mutations but not mutations in other parts of the gene lead to partial nuclear clearance and cytoplasmic accumulation of RBM20. We explored whether augmentation of RBM20 expression using AAV vectors would improve splicing defects and cardiac function in mice carrying missense mutations in RS domain and iPSC-CMs. First, we generated heterozygous knock-in mice (Het KI) carrying a S637A mutation in the RS domain. These animals display significant splicing defects and cardiac structural, functional deficits relative to age matched wild-type animals (WT) similar to that observed in man. Interestingly, longitudinal analysis of the phenotypes in the Het KI mice revealed no worsening of splicing or cardiac structural/functional phenotypes after 14 weeks in age.

Second, we dosed p14 pups and adult Het KI animals with AAV-RBM20 and demonstrated that exogenously expressed RBM20 is correctly localized to the nucleus in the presence of mutant cytoplasmic RBM20. Third, we observed dose dependent restoration of splicing defects in Het KI animals treated with AAV-RBM20. Fourth, prophylactic administration of 1E13vg/kg of AAV-RBM20 in p14 Het KI pups led to improvement of splicing defects in RBM20 target genes and subsequent attenuation of cardiac systolic functional decline. Finally, human iPSC derived cardiomyocytes carrying missense mutations in the RS domain (S635A) and outside the RS domain (V914A) were treated with AAV-RBM20 and demonstrate dose dependent restoration of splicing defects. These data demonstrate that nuclear restoration of RBM20 can provide benefit to disease models with missense mutations in the gene within and outside the RS domain.

P605

EPI-321: a potential cure for Facioscapulohumeral Muscular Dystrophy (FSHD) targeting D4Z4 epigenome

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Facioscapulohumeral muscular dystrophy (FSHD) is one of the most common types of muscular dystrophy with an annual incidence rate of about 1 in 10,000, affecting approximately 800,000 people globally. With no cure available, current therapeutic strategies only involve managing symptoms to improve overall quality of life. Stochastic (mis)expression of disease-causing protein, DUX4, in muscle leads to slow and progressive muscle degeneration through activation of apoptotic and other downstream pathways, which makes the development of cure difficult. *DUX4* gene is encoded at distal region *4q35* chromosome from D4Z4 microsatellite array that in case of FSHD is hypomethylated leading to the brief *DUX4* expression.

At Epic Bio, we leverage our proprietary Gene Expression Modulation System (GEMS) platform to develop a treatment for FSHD that targets the D4Z4 epigenome and permanently suppress *DUX4* expression. Our product, EPI-321, is a single vector AAV serotype *rh74* encoding an ultracompact, catalytically inactive Cas protein (effector) fused to gene-suppressing modulators, under the expression of the muscle specific promoter and a guide RNA targeting *D4Z4* locus. We screened 2 effector proteins, 4 gene expression modulators, and more than 70 different guide RNAs targeting D4Z4 to identify the top candidate, EPI-321. The D4Z4 targeting guide RNA showed no off-target to any known protein coding gene *in silico* when mapped against human genome.

We showed that EPI-321 robustly suppress *DUX4* and DUX4-downstream genes expression in FSHD patient-derived immortalized and primary myoblasts *in vitro*, irrespective of the number of D4Z4 repeats. Functionally, *in vitro* treatment of patients-derived FSHD myoblasts, both primary and immortalized, with EPI-321 decreased rate of apoptotic nuclei assayed by Caspase 3/7 staining. EPI-321 also showed dose-dependent suppression of *DUX4* and DUX4-pathway genes in FSHD patient-derived myoblasts. Mechanistically, EPI-321 showed re-methylation of the D4Z4 targeting locus leading to suppression of *DUX4* expression. Further, we evaluated EPI-321 *in vivo* in a humanized mouse model of FSHD. We showed robust delivery and expression of EPI-321 in the humanized muscle tissue following a single intravenous (IV) dose. In addition to decreasing the DUX4-pathway genes expression, EPI-321 was able to rescue FSHD muscle cell survival by 55% even after four weeks of treatment. Biodistribution study in NHP at 3 months showed high skeletal muscle specific expression of the EPI-321 with very minimal or no expression in any other tissue including liver, kidney, brain, testis, eye etc. at both low and high doses after single IV injection. Importantly, mice and NHP administered with one intravenous dose of EPI-321 showed no serious or adverse events up. We examined clinical signs, blood chemistry, hematology, ECG, Jacketed telemetry, and histopathological, demonstrating the safety of EPI-321.

Taken together, our findings provide evidence for the potential of EPI-321 as a single-administration gene therapy for the treatment of FSHD. We intend to submit an Investigational New Drug (IND) application by the end of 2023 and are looking forward to commencing first-in-human trials in 2024.

P606

MyoScreen™, a discovery platform for candidate selection of muscle-directed AAV gene therapies

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Myotonic Dystrophy type I (DM1) is an autosomal dominant genetic disease characterized by progressive myotonia and weakness of skeletal muscle. The disease is caused by a triplet CUG expansion in the 3' untranslated region of the gene Dystrophia Myotonica Protein Kinase (DMPK). Mutant DMPK mRNA aggregates into nuclear foci, leading to sequestration of Muscleblind like transcription factors (MBNL1) and other transcription factors and RNA binding proteins. A major limitation for therapeutic development is the availability of *in-vitro* model systems that recapitulate the cellular phenotype of DM1 myocytes. In this study we characterized DM1 patient-derived myoblasts and wild type cells cultured with the MyoScreen platform from Cytoo.

MyoScreen is an *in vitro* discovery platform designed for characterizing and screening the efficiency and potency of muscle therapies directly in subject-derived skeletal muscle cells. This physiological culture system reduces assay variability while remaining compatible with conventional high-throughput cell-based assays in addition to more innovative quantitative and functional image-based assays. Under MyoScreen conditions, control healthy myoblasts or infantile, juvenile-onset and adult-onset DM1 subject-derived myoblasts were differentiated on micropatterned plates. Cells were monitored for differentiation using cell surface area, nuclear area, and nuclear fusion. All three subject-derived myocytes displayed 4-6 DMPK foci per nucleus, with no foci detected in control cells. Furthermore, calcium handling was altered in DM1 cells. Splicing was analyzed for nine genes that have previously been demonstrated to have altered splice profiles in DM1 subjects. Splice alterations were recapitulated for 8 of 9 genes tested, with only slight differences between cells from infantile, juvenile, and adult-onset subjects. Finally, myocytes were suitable hosts for AAV transduction, with optimized conditions providing > 80% GFP reporter expression, without an impact on differentiation kinetics.

MyoScreen is an effective in-vitro platform to model neuromuscular disorders. Subject-derived myoblasts, cultured on a micropatterned substrate, have improved differentiation properties, and display molecular phenotypes of DM1. We believe this provides a superior clinically relevant platform for in-vitro evaluation of AAV clinical candidates.

P607

Gene Therapy Mediated Rescue of Acquired Cardiomyopathy and Chronic Heart Failure

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Heart failure (HF) is a major cause of morbidity and mortality worldwide, yet with limited therapeutic options. The majority of patients with HF developed cardiomyopathy from acquired

disease, both ischemic and non-ischemic. Recent evidence indicates that the cardiac bridging integrator 1 (cBIN1) is a structural protein responsible for transverse tubule microdomains which organize the calcium handling machinery important for cardiac function. cBIN1 is reduced in the heart muscle of both acquired and genetic HF, and contributes to negative remodeling of the heart and HF progression. In rodent models, gene therapy with adeno-associated virus 9 (AAV9)-cBIN1 effectively reconstitutes microdomains in heart muscle cells, rescuing heart muscle function and improving mortality. Here we explored the therapeutic efficacy of AAV9-cBIN1 in two large animal models of HF: a canine model of ischemic cardiomyopathy and a Yukatan minipig model of non-ischemic (tachypacing induced) cardiomyopathy. For the canine ischemic animals, healthy adult mongrel dogs (30±3 kg) underwent a lateral thoracotomy followed by left anterior descending artery ligation to induce myocardial infarction. Animals developed progressive dilated cardiomyopathy and chronic ischemic HF over 10 – 16 weeks. When their left ventricular ejection fraction (LVEF) decreased below 40%, animals received a single intramyocardial injection of either adeno-associated virus serotype 9 (AAV9)-cBIN1 gene (n=6) or AAV9-green fluorescent protein (GFP) (Control, n=3) in the LV. For the minipig non-ischemic cohort, 6-7 months old Yucatan minipigs were subjected to continuous right ventricular rapid pacing (RVP) at 170 bpm. After continuous RVP for 5.7 ± 1.0 weeks (N=10), minipigs developed dilated cardiomyopathy and reduced ejection fraction. Once LVEF ≤40%, pigs were randomized to receive intravenous AAV9-cBIN1 or control (AAV9-GFP or PBS). Pacing was continued throughout the study period. Results in both models were notable for substantial improvements in LVEF, reductions in cardiac chamber size, and improved mortality. A single dose of cBIN1 gene therapy has therapeutic efficacy in minipigs for as long as six months before the animals were terminated. cBIN1 is a higher order regulator of excitation-contraction machinery in cardiomyocytes, and only small (20-30%) reductions in the protein occur with natural HF progression. Therefore, low dose replacement is sufficient for efficacy. Therapeutic dosing for cBIN1 is 1/10th of that of current gene therapies, minimizing toxic off-target effects associated with AAV administration. In conclusion, using large-animal pre-clinical models, low dose cBIN1 gene therapy is efficacious in causing partial reversal of both ischemic and non-ischemic dilated cardiomyopathy, substantially increases LVEF, and improves mortality in chronic HF.

P608

Gene therapy for Friedreich Ataxia: evaluation of regulatory elements to control frataxin expression

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Friedreich's ataxia (FA) is a rare genetic disorder caused by deficiency of frataxin, a mitochondrial protein implicated in the synthesis of essential iron-sulfur cluster cofactors (Fe-S). Preclinical studies in mice have shown that AAV-mediated gene replacement is able to correct disease-relevant cardiac and neurological phenotypes. However, we and others have shown that overexpression of frataxin is toxic, and this toxicity is dependent on frataxin's association with the Fe-S biosynthetic machinery. To overcome toxicity and control frataxin expression, we designed a transgene expression cassette including regulatory sequences (RegFXN) and tested it in cells, WT mice, and a cardiac FA mouse model.

When the construct was tested in inducible knock out frataxin cells, the Fe-S deficiency induced by frataxin removal was efficiently prevented. To test if this construct could control the levels of

expression *in vivo*, we injected WT mice and a cardiac FA model, by systemic administration of AAV9-RegFXN constructs. Inclusion of the Reg sequences effectively regulated expression of frataxin in heart and dorsal root ganglia, among other tissues, in WT animals. No body weight loss or signs of liver toxicity were observed with doses up to 6E13 vg/kg in both WT and FA mouse models. When tested in a cardiac FA model, AAV9-RegFXN treatment led to extension of survival, and correction of functional and molecular phenotypes. These included several cardiac parameters such as ejection fraction, fractional shortening, and biomarkers, as well as correction of Fe-S deficiency in heart and muscle. Complete functional cardiac correction was observed with doses of AAV9-RegFXN as low as 1E13 vg/kg.

In conclusion, we identified regulatory sequences that effectively regulate frataxin expression *in vitro* and *in vivo*. The designed regulation system provides a novel and promising means for the development of safe and effective FA gene therapy approaches.

P609

Testing angiogenic transgenes in endothelial-specific lentiviral vectors

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Despite advances in pharmacologic care, ischemic heart disease remains the leading cause of global mortality over the last 15 years necessitating further development of adjunct therapies or alternative approaches. To this end gene therapy treatments utilizing viral vector delivery of angiogenic factors were developed but have yet to yield success in clinical trials. Our recent work with novel cell-specific viral vectors and therapeutic transgenes supports a novel therapeutic strategy. We hypothesize that in the ischemic heart, cell-specific transgene vectors can increase angiogenesis to promote effective tissue repair. To this end we have already developed an endothelial-specific vector expressing a green fluorescence reporter gene (LV-E2-GFP). This development was based on the use of endothelial-specific super-enhancers to derive regulatory elements that can activate gene expression specifically in endothelial cells. In the current work we test this vector with different transgenes for activation of angiogenesis. Using gene expression and functional assays we demonstrate the activation of angiogenic transgenes from these vectors in endothelial cells and demonstrate their effect on endothelial sprouting and tube formation. In addition, ongoing *in vivo* testing of these vectors in mouse hind-limb and in a mouse myocardial infarction model we assess the effectiveness, expression and biodistribution of these vectors. These studies may bring us a step further towards effective angiogenic gene therapy for the treatment of ischemic diseases.

P610

Improving intramyocardial gene transfer methods

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Despite current therapeutics, coronary artery disease is a significant cause of mortality and morbidity worldwide. Therapeutic angiogenesis induced by gene therapy is a promising treatment for patients with severe coronary artery disease. The gene therapy can be carried out by local intramyocardial injections.

Large animal models are needed to evaluate the safety and efficacy of intramyocardial gene transfers. We use an ischemic porcine animal model done by occluding the left ascending coronary artery with a bottleneck-shaped stent. The occlusion leads to an ischemic area in the anterior and septal walls of the left ventricle, mimicking a myocardial infarction caused by coronary artery disease.

Our intramyocardial injections are done percutaneously. We introduce an injection catheter via a femoral sheath to the aorta and through the aortic valve under fluoroscopic guidance. Once in the left ventricle, the injection catheter can be navigated in the ventricle and positioned against the wall. We then introduce the needle and inject our treatment solution to the myocardium slowly, e.g. 0,1ml in 15sec and count another 5 seconds to avoid refractory flow before removing the needle.

The injection locations in the target area can be verified by two techniques: first, we can verify the position of the injection catheter by imaging it from two different angles, one showing the lateral and vertical position of the catheter and another in 90 degree angle to that to show the positioning in relation to the anterior wall. Another technique is to map the left ventricle with an electromagnetic mapping system and position the injections in the ventricle according to the 3D map. With this technique, there is the advantage of evaluating the target site, targeting the hypoxic, yet viable tissue. Injection catheters may also be combined with CT dataset heart navigation to determine the exact position of the infarction and the gene transfer injections in the heart.

P611

Rescue of dysferlin-deficient muscular dystrophy by gene-edited primary muscle stem cells

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Muscular dystrophies (MD) are a group of monogenic, severely debilitating and untreatable diseases causing progressive and irreversible degeneration of skeletal muscle. In this study, we targeted a frequent, loss-of-function founder frameshift mutation in *DYSF* exon 44 causing

dysferlin-deficient MD. We found that mRNA-mediated delivery of SpCas9 in combination with a mutation-specific sgRNA to primary muscle stem cells (MuSC) from two homozygous patients consistently led to >60% exon 44 re-framing, rescuing full-length and functional dysferlin protein. A new mouse model harboring a humanized *Dysf* exon 44 with the founder mutation, *hEx44mut*, recapitulated the patients' phenotype and an identical re-framing outcome in primary MuSC. Finally, gene-edited murine MuSC regenerated muscle and rescued dysferlin when transplanted back into *hEx44mut* hosts. Our findings show for the first time a somatic CRISPR therapy for dysferlin-deficiency and demonstrate *in vivo* proof-of-concept for the autologous use of gene-edited primary MuSC in cell replacement therapies to treat MD.

P612

In vivo AAV based gene therapy reduces cardiac conduction defects in TMEM43 mediated arrhythmogenic cardiomyopathy

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TMEM43 is a transmembrane protein mainly located in the inner nuclear membrane of cells. Although its function is still unknown, the mutation pS358L leads to the most aggressive subtype of arrhythmogenic cardiomyopathy (ACM), known as TMEM43-ACM or ACM type 5. TMEM43-ACM is an autosomal dominant fully penetrant disease characterised by chamber dilation and a progressive dystrophy of the ventricular myocardium, leading to life-threatening ventricular arrhythmias and sudden cardiac death.

Unpublished data from our lab shows an interaction between the wild type (WT) and pS358L forms of TMEM43, suggesting that WT-TMEM43 may counteract the detrimental effects of the mutant protein by forming a heterodimer. Consequently, we propose that overexpressing WT-TMEM43 could reduce the toxicity of S358L-TMEM43. Using our previously reported transgenic mouse models overexpressing either WT- or pS358L-TMEM43, we generated a double transgenic mouse (DT) that overexpresses both forms of the protein. This study demonstrated that WT-TMEM43 overexpression improves systolic function, reduces cardiac conduction defects and slows down TMEM43-ACM progression, significantly increasing the lifespan of DT mice compared to mutant mice.

To enhance the translational potential of this approach, we have developed a self-complementary adeno-associated viral (scAAV) vector expressing an optimized version of WT-TMEM43 under the control of the chicken cardiac troponin T (cTnT) promoter. Intravenous delivery of this vector results in high expression of WT-TMEM43 in the heart of mice. Preliminary data from AAV-injected TMEM43mut mice indicate reduced QRS and P-wave durations, as well as increased QRS amplitude observed by electrocardiography, indicating an improved cardiac conduction compared to untreated TMEM43mut mice.

The preclinical data presented here demonstrate the potential of our optimized WT-TMEM43 gene therapy vector in preventing cardiac conduction defects in TMEM43-ACM mice. This therapy offers a promising alternative to palliative treatments for patients suffering from this highly lethal disease.

P613

An AAV gene therapy for *LMNA* dilated cardiomyopathy via disruption of the LINC complex

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Dilated cardiomyopathy (DCM) can arise from genetic mutations and are thus an attractive disease target for adeno-associated virus (AAV) gene therapies. However, a significant proportion of DCM mutations are autosomal dominant and gain-of-function, precluding gene replacement therapy. One of the most frequent genetic causes of DCM, due to mutations in the *LMNA* gene, falls into this category.

LMNA encodes lamin A/C, an intermediate filament protein that forms a filamentous network underlying the nuclear envelope, known as the nuclear lamina. The nuclear lamina is thought to protect the nucleus from cytoskeletal force transmission, and mutations in *LMNA* weaken this protective network. This is thought to lead to nuclear envelope rupture and consequently cell and tissue damage. The linker of nucleoskeleton and cytoskeleton (LINC) complex spans the nuclear envelope and physically couples the cytoskeleton to the nucleus, thereby mediating cytoskeletal force transmission. We hypothesized that reducing these forces could improve *LMNA*-linked pathology. Indeed, using AAV to drive cardiac expression of a LINC complex disrupting transgene, GSLA01, in cardiomyocytes, ameliorates cardiac pathology in a *LMNA* DCM mouse model.

As part of lead optimization efforts, we investigated structure-function relationships for GSLA01 by generating a series of truncations of the full-length transgene. Using in vitro assays, we determined that most truncations examined could disrupt the LINC complex. AAVs were then generated harbouring selected truncations of GSLA01, and delivered to *LMNA* DCM mice where cardiac-specific deletion of Lmna was induced with tamoxifen. The lifespan of *LMNA* DCM mice was extended from 39 to ~170 days using full-length GSLA01, while other truncations performed on par or better, exceeding 250 days for certain constructs.

Disrupting the LINC complex using a variety of GSLA01 variants appears to be a robust strategy for treating *LMNA* DCM, and addresses the limitations of gene replacement therapy in a disease characterized by gain-of-function mutations.

P614

Development in vitro models and viral vectors and for gene replacement therapy of calpainopathy (LGMD2A/R1)

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Calpainopathies are a subgroup of Limb Girdle Muscular dystrophies (LGMD), caused by mutations in the calpain 3 gene (*CAPN3*). The disease is characterized by progressive, symmetrical

weakness of the proximal limb and girdle muscles. For gene therapeutic treatment approaches, vectors based on the capsids of muscle transducing AAV serotypes, as well as 3rd generation Adenoviruses (HCAdV's) are tested preclinically. In this study, we established two human cell culture disease models. We used patient derived immortalised myotubes as well as IPS cell derived human muscle cells with defined CAPN3 point mutations and an applied advanced protocols based on electrical pulse stimulation (EPS) to evoke differentiation and sarcomere formation in 2D cell culture. For analysis of viral vector mediated CAPN3 gene replacement in differentiated myotubes vectors based on HCAdV's (serotype 5), AAV9 and AAVmyo (muscle specific AAV-based vector) encoding an identical *CAPN3* cDNA fused to a GFP gene separated by a 2A peptide signal under control of the MHCK7 promotor. All vectors genomes were assembled and vectors were generated and produced to high titers. We used these vectors to transduce differentiated CAPN3 deficient myotubes compared to healthy immortalized controls or CRIPR/Cas9 corrected IPS cell derived myotubes. CAPN3 gene transfer and expression was analyzed by RT-qPCR, Western blot and fluorescent microscopy. Surprisingly HCAdV mediated gene transfer didn't increase levels of Calpain3 whereas a GFP expressing HCAdV revealed robust gene expression and a bright GFP-signal in the myotubes indicating that CAPN3 expression from the HCAdV genome was impaired in this setting. AAV mediated gene transfer of the human full length *CAPN3* cDNA resulted in enhanced *CAPN3* gene expression *in vitro* and had compensatory effects on protein levels in the two different Capn3-disease models. This *in vitro* study provides novel preclinical models that helps studying potential treatment options for Calpainopathies and provides insights helping to choose ideal gene transfer vehicle and or transgene expression setup for future translation into in vivo studies regarding tissue specificity and expression strength.

P615

Adenovector-mediated reduction of VEGFR2 signalling uncovers novel inflammatory effects of VEGF-D in the gut

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Small intestinal blood and lymphatic vascular systems have key roles in transporting nutrients and in effective immune defence against cancers and ingested pathogens. Changes in intestinal vasculature have been associated with conditions like inflammatory bowel diseases (IBD), where intestinal homeostasis is disbalanced and chronic inflammation takes over. Lately, several new signalling proteins have been found to affect lymphatic and blood capillary development and function in the intestine. Many of these molecules belong to the Vascular Endothelial Growth Factor (VEGF) protein family or to their receptors (VEGFRs). VEGF-D has been shown to affect both angiogenesis and lymphangiogenesis since it binds both VEGFR2 and VEGFR3 and it is produced in many tissues including lungs, skin, heart, skeletal muscle, and the gastrointestinal tract. However, relatively little is known about its functions and the effects of VEGF-D on intestinal vasculature remain unclear. Additionally, the combined effect of altering both intestinal VEGF-D levels and VEGF receptors has not yet been studied.

Here, we use the combination of genetically modified mouse models and adenoviral gene transfers as tools to uncover the intestinal effects of VEGF-D and its combined effects with other VEGFs. We examine the intestinal phenotype of VEGF-D deficient mice (*Vegfd*$^{-/-}$) and further, by

using adenoviral vectors, we explore the effects of binding the two most potential compensators of VEGF-D loss (VEGF-A & VEGF-C) by soluble VEGFR2 (Ad-sVEGFR2).

Characterisation of the adenovector was done by sequencing, and efficacy was confirmed *in vitro*. Mice were given several doses of either Ad-sVEGFR2 or Ad-CMV (control) *i.v.* By using immunohistology, clinical chemistry and gene expression assays, we determine effects of VEGF-D on intestinal vasculature, and we show dose-dependent effects of adenovector-mediated inhibition of VEGFR2 ligands on intestinal homeostasis. Inhibition of signalling through VEGFR2 leads to differential damage and inflammatory response in the intestine depending on VEGF-D availability.

These results mark VEGF-D as a previously unknown mediator of GI tract homeostasis. However, loss of VEGF-D can be partially compensated by other VEGFs. In the future, measuring VEGF-D levels of patients presenting with IBD or other gastrointestinal diseases related to inflammatory pathways could be of interest.

P617

Use of capillary Western immunoassay for quantification of micro-Dystrophin transgene product after rAAV-based gene therapy

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Duchenne Muscular Dystrophy (DMD) is a fatal X-linked degenerative neuromuscular disease caused by mutations in the DMD gene coding for dystrophin. Gene therapy using optimized micro-dystrophin (MD) transgenes and recombinant Adeno-Associated Virus (rAAV) vectors hold great promise. Since 2018, four clinical trials using this strategy have been launched. MD protein expression evaluation in skeletal and cardiac muscles in preclinical and clinical subjects dosed with rAAV-MD is an important factor to measure treatment efficacy of the gene therapy. Western blotting is considered as the gold standard for MD protein detection and quantification. Although this is a proven technique, conventional Western blotting is time consuming and suffers from poor reproducibility due to multiple manual steps as protein loading, membrane transfer, immunoprobing and protein detection. To counteract these limitations, capillary Western assay system (also known as Simple Western) has been developed. In Simple Western analysis, all steps following sample preparation are fully automated, requires only very low amounts of sample and antibody, and can precisely measure proteins at nanogram levels. Here, we evaluated the application of the Simple Western immunoassay (Jess™, from Biotechne) method for MD protein detection and quantification in tissues of DMD^{mdx} rats injected with a rAAV2/9-MD vector. Our data showed that MD quantification by Jess is highly sensitive, reproducible and quantitative over a large dynamic range compared to traditional Western blotting. This study demonstrates the high potential of the Simple Western technology for MD protein expression evaluation in subjects dosed with rAAV-MD in preclinical and clinical studies.

P618

Preliminary results from a prospective, multicentric, follow up standardized cohort to assess natural history of Duchenne muscular dystrophy.

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Duchenne muscular dystrophy (DMD) is a rare monogenic neuromuscular disease characterized by progressive muscle damage beginning before birth with slowed physical development resulting in functional decline from age 6, loss of ambulation between 8- 15 years of age, and early mortality. Multiple therapies aimed at delaying or stabilizing disease progression are in development. However, the heterogenous clinical course of the disease makes it crucial to understand baseline evolution of assessments to be measured in clinical trials.

We are conducting a multicentric, international study in ambulant DMD boys to assess longitudinally measurements prior to enrollment in a gene therapy study. Enrollment included patients aged 5-9 years old who achieved a North Star Ambulatory Assessment (NSAA) score ≥ 18 at inclusion. All patients were assessed using NSAA, timed function tests, MyoTools (arm muscles force tests), cardiac and respiratory function tests every 6 months. Functional and muscles force tests assessors were trained and each patient is evaluated by the same assessor throughout the study whenever possible. This analysis includes 48 patients with ≥ 6 months follow up (mean= 13.5 months) from France and UK. Descriptive statistics by age group ([4-6[; [6-7[; [7-8[or [8-10] years old at inclusion), correlation analyses (Spearman) and Standardized Response Mean (SRM) of these endpoints were performed.

At inclusion, patient's mean age was 7.10 ± 1.37 years and mean weight was 21.76 (±4.03) kg, 95.7% were receiving oral steroid treatment (mean duration:14 months). The mean NSAA score was 25.52 ± 4.24, and mean time to Raise from Floor (TTRFF) was 4.43 ± 1.51s, with NSAA means being similar between patients < 7 and ≥7 years of age. Mean Forced Vital Capacity (FVC) and Forced Expiratory Volume in one second (FEV1) were 90.35±18.85 % predicted and 92.83±17.72 % predicted. Physical function tests scores were mostly stable or improving over time in younger patients, whereas they tend to decrease in older patients: e.g., NSAA slope of -0.07 (95% CI: [-1.79; 1.65] p=0.9341) in patients < 7yo and -3.19 (95% CI: [-5.03; -1.35] p=0.0011) in those ≥ 7yo at 12 months. The same trend was observed in pulmonary tests, suggesting early respiratory muscle decline at time of lower limb decline. NSAA, Time to 10 Meters Walk/Run Test (10MWT) and TTRFF correlated well between each other (Rho between 0.75 and 0.81). Arm force measurements (Myopinch and Myogrip) remained stable during follow-up and correlated poorly with other functional tests, consistent with other reports of the trajectories of upper limb decline occurring later in disease progression.

The findings of this study with a standardized follow-up and outcome measurements reinforce the need to include multi-measures for accurate outcome assessment in clinical trials with DMD, as different assessments decline at different rates and at different timepoints. It also supports to use NSAA as primary endpoint in this younger ambulatory population. These early results suggest that the monitoring of respiratory function may be useful to detect early divergence from expected trajectories for study-drug evaluation. More longitudinal data are being collected for a more robust and comprehensive analysis.

P619

Investigating the role of ADAMTS18 in angiogenesis for potential angiogenic gene therapy

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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 remodeling. 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.

P620

Multilevel assessment of CRISPR/Cas9 mediated silencing of a pathogenic variant in *COL6A1* as a potential therapeutic strategy for collagen VI-related muscular dystrophies.

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Collagen VI-related disorders (COL6-RD) represent a severe form of congenital muscular disease for which there is no treatment. Here we used patient-derived fibroblasts bearing the missense c.877G>A, p. 293Gly>Arg dominant negative mutation as a cellular model of COL6-RD. We tested the potential of CRISPR/Cas9-based genome editing to silence or correct (using a donor template) the mutant allele in the dermal fibroblasts. To this end, it is essential to optimice bioinformatic and molecular tools to precisely evaluate the efficacy and specificity of this therapeutic strategy, among others that may arise in the future in the field of gene editing. Firstly, at a genomic level, evaluation of gene-edited cells by next generation sequencing coupled to Mosaic Finder Allelic variaton analysis revealed that correction of the mutant allele by homologous-directed repair occurred at a frequency lower than 1%, while in most cases what was observed was a specific editing of the mutant allele mediated by NHEJ. We further confirmed at the transcriptional level the reduction in the expression of the mutant allele using droplet digital PCR with allele specific probes. It was observed that the expression of the wild type allele was not affected, confirming the specificity towards the pathogenic allele of this strategy. Next, we analyzed the potency of editing at the protein level, studying the structure of the collagen VI microfibrillar network in the extracellular matrix by immunofluorescence and high resolution confocal microscopy. Analysis of various quantitative parameters demonstrated recovery of collagen VI.

Furthermore, we combined stimulated emission-depletion (STED) super-resolution microscopy with soft-cry X-ray tomography analysis to characterize at the cellular and functional level healthy control and patient-derived fibroblasts. Cultured patient-derived fibroblasts exhibit unusual extracellular collagen VI network, and the intracellular hallmark of the pathology such as mitochondria and Golgi fragmentation. We further revealed a novel intracellular alteration characterized by endo-lysosomal accumulation, and uncovered the molecular mechanism behind it; in particular, we detected higher phosphorylation levels of the collagen VI receptor CMG2, thus boosting the endocytic pathways and resulting in endosomes and lysosomes accumulation in mutant cells. Importantly, gene editing was able to rescue the different intracellular hallmarks observed in the patient-derived fibroblasts.

In summary, in this work we have fine-tuned a wide variety of tools to assess the efficacy of CRISPR/Cas-mediated gene editing strategies at the genomic, transcriptional, protein and cellular level. This is essential to provide robust evidence and data when moving towards the use of these strategies in clinical trials and ultimately in clinical practice for the treatment of patients.

P621

Enhancement of porcine fibroblast migration by macrophages treated with secretome from cardiosphere-derived cells, with no impact on collagen production

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After an acute myocardial infarction (AMI), an inflammatory response is triggered, and macrophages are mobilised towards the infarcted myocardium. Their main function is to eliminate necrotic and apoptotic cardiomyocytes and facilitate wound repair.

In the later stages of cardiac regeneration, myofibroblasts initiate a healing response, including the formation of collagen fibres. This process is vital to maintain the structural integrity of the infarcted ventricle. However, while initial reparative fibrosis is necessary to prevent ventricular wall rupture, excessive fibrosis caused by excessive secretion and deposition of extracellular matrix within infarcted areas can result in oversized scar tissue.

Macrophages, recruited in the heart after MI, have a dual role as key inflammatory components and central regulators of cardiac injury. Therefore, it is crucial to regulate fibrosis to improve the prognosis of AMI. In this context, the administration of secretomes from mesenchymal stromal cells has been suggested as a promising therapeutic approach, as they modulate the activation state of macrophages under *in vitro* and *in vivo* conditions.

Pigs are widely used as animal models in the field of cardiovascular diseases, due to their similarity to the human heart. Therefore, we used porcine macrophages to study the antifibrotic capacity of the secretome of porcine cardiosphere-derived cells (S-CDCs) in vitro.

Porcine macrophages were isolated from the peripheral blood of healthy pigs and polarized towards M2 by adding hM-CSF to the medium. On day 7 of culture, after 24 hours of stimulation with IL-4 and treatment with S-CDCs, the cell culture medium was replaced with M199 medium + 1% ITS. After 24 hours, the conditioned medium was collected and the protein concentration was quantified.

To determine whether S-CDC treatment inhibited fibrosis, porcine cardiac fibroblasts were cultured with 100mg/ml of conditioned medium released from treated and untreated M2.

S-CDC-treated M2 macrophages slightly stimulated wound healing and cardiac fibroblast migration; however, qPCR analysis showed that it decreased the expression of fibrotic genes such as *COL I*, *COL III*, *FGF1*, and *ACTA2*.

In conclusion, S-CDC treatment could regulate fibrosis and improve the prognosis of AMI, providing new approaches for the development of new treatment strategies.

P622

Detection of residual stem cell contamination in cell therapy products

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Stem cells have been increasingly explored as an attractive source for cell therapy due to their ability to proliferate rapidly *in vitro* and, in theory, differentiate into any cell type. However, a relevant major safety concern is the possible contamination of the final therapeutic product with undifferentiated pluripotent cells which can lead to teratoma formation. This is a safety risk that needs to be mitigated for a successful translation into the clinic. The commonly used high efficiency culture (HEC) assay has been proven robust and highly sensitive for detection of residual stem cells. We used different spike-in concentrations to evaluate the assay's limit of detection of contaminating ESCs in cultures of differentiated cells. Furthermore, we performed critical assay optimization by comparing three culture systems to identify the conditions that best support survival of the ESCs. Our results confirm the HEC assay to be highly sensitive and demonstrate how the microenvironmental differences between the culture systems tested can affect the limit of detection of the assay. Comparatively, our *in vivo* studies with subcutaneous injection of the same cells were less sensitive in predicting teratoma forming units. In parallel, we present preliminary data on the identification of suitable markers of both ESCs and iPSCs for use with the digital droplet PCR as a complementary step for detecting undifferentiated stem cells. In conclusion, we have optimized the HEC assay to be a highly sensitive *in vitro* alternative for the detection of the presence of ESCs in a cell therapy product.

P623

A Muscle Targeted Gene Therapy for Kennedys Disease

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Spinal & Bulbar Muscular Atrophy (SBMA) is a progressive neuromuscular disorder characterised by loss of lower motor neurons within the spinal cord and brainstem and skeletal muscle atrophy. All SBMA patients suffer from the same abnormal CAG repeat expansion in exon 1 of the Androgen Receptor gene, with expansions of greater than 38 repeats resulting in significant disability affecting mobility, speech, swallowing and breathing. As yet, there are no effective treatments available for SBMA patients. Our aim is to develop a therapeutic to reduce the expression of Androgen Receptor specifically in skeletal muscles. To date we have 1) established a strategy for delivering therapeutics specifically to skeletal muscle in vivo by exploiting a recently described AAV vector known as MyoAAV. and 2) developed a system for reducing the expression of Androgen Receptor in vitro. This system we have validated utilises Cas13d; a small ribonuclease that can be programmed to bind and cleave RNA using a guide RNA. We have validated multiple guide RNAs that target the Cas13d to AR and significantly reduce Androgen Receptor expression in vitro.

Our strategies form a crucial platform from which a viable therapeutic can be built. Future delivery of our therapeutic to both wild type and SBMA mouse models will provide key information as to the extent of AR knockdown required for the progression of the disease and its symptoms to be

altered; something that is not currently well defined. Furthermore, as SBMA is a monogenic disease, with all cases resulting from the same abnormal CAG repeat expansion in the AR gene, our innovative therapy approach would be applicable to all SBMA patients.

P624

Healthy primary pericytes moderate disease characteristics in a 2D myogenic cell model for myotonic dystrophy

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Myotonic dystrophy type 1 (DM1) is the most common form of adult muscular dystrophy caused by an expanded CTG repeat in *DMPK* gene. This toxic expanded repeat leads to the creation of repeat RNA-containing nuclear foci, which in turn sequesters MBNL1 protein, leading to a cascade of mis-spliced proteins. We aim to set up a personalized cell therapy to alleviate the muscular phenotype by addition of pericytes; a cell type that can be isolated from post-natal tissue and has the unique capacity to travel through the bloodstream to fuse together with damaged muscle, giving rise to the possibility of an intra-arterial cell therapy approach. Previously, we isolated pericytes, a distinct class of muscle progenitor cells, from quadriceps muscle of six DM1 patients and two healthy individuals.

Here, we report on a detailed analysis of the myogenic behavior and the effect differentiation on pericytes and the effect of pericytes on DM1 hallmarks in differentiating 2D co-cultures with DM1 myoblasts. During differentiation of pericytes to myotubes the increase in myogenic differentiation marker *MHC* coincided with higher expression of *DMPK* quantified by RT-qPCR. Quantification of RNA foci in nuclei of MHC-negative cells and in MHC-positive myotubes showed a significant increase in RNA- and MBNL1-positive foci for patient derived pericytes, but not for control pericyte-derived myotubes.

Co-cultures of primary pericytes with DM1 myoblasts were performed to determine myogenic fusion capacity. We found no significant difference between the fusion capacity of healthy and disease pericyte populations. However, when we look at DM1 disease characteristics we found a decrease in the amount of RNA foci and an increase in foci size in DM1 nuclei concomitant with the percentage of healthy pericytes contained in the myotubes. When looking at free nuclear MBNL1 as a proxy for disease severity, we found that mixed myotubes made up of >21% healthy pericyte nuclei displayed a significantly higher free MBNL1 concentration in DM1 nuclei after 5 days in culture (C1: $p < 0.0001$) while for our other healthy pericyte line this was the case at >31% (C2: $p = 0.00179$).

We showed that differentiated DM1 pericyte cultures exhibit disease hallmarks such as RNA- and MBNL1 foci, although the disease state of pericytes does not influence their fusion capacity in 2D co-cultures. Furthermore, we show a decreased amount of RNA foci and increased amount of free MBNL1 found in DM1 nuclei in mixed co-cultures with healthy pericytes. This builds the foundation for our cell therapy approach to alleviate the muscle phenotype in DM1 patients.

P626

Allele-specific epigenome editing of *COL6A2* as potential therapeutic approach for COLVI-CMD

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Collagen VI congenital muscular dystrophy (COL VI-CMD) is a rare neuromuscular spectrum disorder that affects around five in one million. The extreme ends of the spectrum are called Bethlem Myopathy (BM, mild and late-onset, OMIM #158810) and Ullrich congenital muscular dystrophy (UCMD, severe and early-onset, OMIM #254090) and are connected by intermediate phenotypes. Common symptoms include hypermobility , contractures, respiratory insufficiency and skin abnormalities. Genetically the disease is caused by mutations in *COL6A1*, *COL6A2*, *COL63* and in very rare cases also in *COL12A1*. Unfortunately, there is no treatment available yet.

There are different types of mutations and we currently focus our research on dominant-negative Glycine-substitutions in *COL6A2*. These pathogenic variants are located in so called triple-helical repeats (Gly-X-Y) and they lead to a dramatic decrease in normally formed Collagen VI tetramers that can be secreted into the extracellular matrix to support the muscle. Since haploinsuffiency is tolerated, the general aim of a therapeutic approach is to knockdown the pathogenic allele specifically to increase the amount of correct tetramers. Considering the rareness of the disease itself and its causing variants, that are almost private, mutation-specific approaches are difficult to proceed. Therefore the aim of our project is to investigate a therapeutic approach that is mutation-independent but still allele-specific.

Epigenome editing can be utilized to precisely regulate gene expression and CRISPRoff is a fairly new method that can achieve that. It uses a dCas9 that is coupled to methyltransferase domains and when it is guided to regulatory elements of a target gene it methylates those regions leading to a transcriptional repression of the target gene expression. Before the design of allele-specific guideRNAs, a 57kb region including *COL6A2*'s regulatory elements and the gene itself were sequenced using nanopore sequencing and a method called Cas9 enrichment. Common variants were called and phased for two patient-derived fibroblast cell lines that exhibit different heterozygous disease-causing point mutations. Shared heterozygous common variants in the promotor were identified and guideRNAs were designed. Unfortunately, primary fibroblasts are notoriously difficult to transfect but using engineered virus-like particles we were able to show that it is possible to knockdown the pathogenic allele specifically without being mutation-specific.

P627

Comparative transcriptomic analysis of the effects of menstrual blood-derived stromal cells' secretome in remote infarcted myocardial tissue: basal vs priming conditions

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A blockage in the coronary artery leads to insufficient blood flow to a region of the heart, resulting in ischemia and subsequent cell death. These ischemic insults have a significant impact on structural and functional changes in remote myocardium. Different therapies and strategies are being developed to mitigate cardiomyocyte damage and prevent cardiac remodelling. In this scenario, secretome-based therapies using priming cell strategies are a promising approach. In this study, we aimed to compare the effects of the secretome from TNFa- and INFg-primed menstrual blood derived-stromal cells (S-MenSCs*) with secretome from menstrual blood derived-stromal cells under basal conditions (S-MenSCs) in an ischemia-reperfusion myocardial injury model.

Balloon occlusion of the mid-left anterior descending coronary artery was performed during 90 minutes in 8 Large-White pigs. 72 hours after infarct induction, the S-MenSCs* (n=4) and S-MenSCs (n=4) were administered intrapericardially. Seven days after therapy, the pigs were euthanised and tissue was collected from the remote area of the myocardium for transcriptomic analysis.

Comparisons between myocardial remote tissues treated with S-MenSCs* and S-MenSCs revealed different expression patterns. Functional enrichment analysis showed a Gene Ontology enrichment term related to protein folding (GO:0006457) including 8 differentially expressed genes in the genes grouped in the down-expressed cluster of S-MenSCs* treatment.

Protein folding plays a critical role in the development and progression of myocardial infarction. Ischemia disrupts the normal functioning of the endoplasmic reticulum, leading to the accumulation of misfolded proteins and protein aggregation, which further worsen tissue damage. Within this mechanism, chaperones, which act as quality control proteins, are crucial in cardiac protection.

In conclusion, the downregulation of protein expression related to protein folding, observed after the administration of S-MenSCs* compared to S-MenSCs, suggests that priming decreases the cardioprotective effect of secretomes in remote myocardium.

P628

Transcriptional optimization of cardiac gene therapy

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Gene therapy for cardiovascular diseases has seen several clinical trials, among others aimed at heart failure. However, these trials have not had satisfactory outcomes, despite encouraging pre-clinical outcomes in the treatment of heart failure. The challenges in cardiac gene therapy development have been attributed to several factors, from transgene ineffectiveness to immune responses and lower-than-expected expression. These hurdles call for optimization of all aspects of gene therapy interventions. In this project, our aim is to optimize the promoter of cardiac gene therapy vectors beyond the current state of the art.

Based on transcriptome data (RNA-seq) and chromatin accessibility data (ATAC-seq), we selected candidate promoter regions from a set of highly expressed and accessible cardiac genes, and have tested these in vitro in a cardiomyocyte-like cell line using luciferase assays. A further selected set was produced as AAV9 luciferase reporter vectors, which were systemically injected at 5*10^11 vg/mouse. After 4 weeks, luciferase expression was quantified in tissue homogenates of the left ventricle, right ventricle, atria and liver.

In vitro screenings revealed 8 novel promoter candidates with significantly higher expression in the cardiomyocyte-like cell line compared to the benchmark TNNT2 promoter, while all were significantly weaker compared to the non-tissue-specific CMV promoter. Subsequent *in vivo* injection of an initial subset of 6 novel promoter candidates, together with benchmark CMV and TNNT2 promoters, revealed none of the novel candidates was stronger, nor more specific than the benchmark TNNT2 promoter. An additional finding was the occurrence of vector build-up in the liver compared to other organs, while other work has also shown leaky expression of the TNNT2 promoter in this organ. These off-target effects may necessitate further optimization of the capsid and transcriptional properties of cardiac-targeted gene therapy vectors. Current work is focused on further optimizing the promoters identified in this screen and testing of new promoter design strategies. Comparative analysis of *in vitro* assays in the cardiomyocyte-like cell line and neonatal rat ventricular myocytes also showed the neonatal rat ventricular myocytes faithfully predicting *in vivo* mouse activity.

P629

Functional Potency Assay Development for the *in vitro* Evaluation of Adeno-Associated Virus Delivered Microdystrophin Biological Activity

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Developing a quantitative *in vitro* potency assay for Duchenne muscular dystrophy (DMD) has proven difficult and has led to challenges across the various stages of clinical development. A vital

component of the µDys/dystrophin mechanism of action is that it forms a complex with various binding partners at the sarcolemma (DAPC), which protects the muscle against contraction-induced damage. Among these binding partners, β-dystroglycan (β-DG), an integral membrane protein, and neuronal nitric oxide synthase (nNOS), which generates NO at the membrane surface, are both thought to be critical components of a functional DAPC. Therefore, we have focused on these two targets for development of a µDys functional potency assay.

To address µDys/β-DG binding interactions *in vitro*, we adapted a sandwich ELISA to measure β-DG protein binding to µDys in lysates of transduced DMD^{KO} myotubes. The ELISA showed a dose response consistent with µDys protein expression within a 1.5-log dynamic range. The assay also demonstrated strong linearity and low variability.

We also adapted the Duolink® Proximity Ligation Assay (PLA) method to allow for *in situ* detection of µDys/nNOS interactions in transduced DMD^{KO} myotubes. The µDys/nNOS PLA displayed a dose response over a 1.5-log range as well, with strong linearity and low inter-assay variability. Both the µDys/β-DG ELISA and µDys/nNOS PLA were successfully applied to tissue lysates and sections, respectively.

Furthermore, rAAV-µDys reduced the release of skeletal troponin I and provided protection against stimulation-induced creatine kinase release, further supporting improved membrane integrity via a functional DAPC. In summary, we established robust *in vitro* methods to characterize mDys activity through DAPC interactions that support the possible use of either method as a possible functional potency assay.

P630

Modeling myocardial infarction recovery in porcine heart: ischemia-reperfusion injury and inflammatory tissue environment

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Cardiovascular diseases are among the leading causes of death and hospitalization worldwide. Despite advances in treatment, there still exists a considerable number of patients with unmet clinical needs, and thus developing new therapeutic strategies is critical. Advanced coronary artery disease is most often the culprit behind acute cardiac events, such as myocardial infarction, which is brought on by a sudden total obstruction in the coronary arteries, cutting off the blood supply to the heart and resulting in ischemic damage. Restoration of blood flow paradoxically intensifies the injury by promoting oxidative stress and excessive neutrophil infiltration into the myocardium. The first three days post-infarction cover the peak of the inflammatory period, during which leukocyte influx is at its highest and enzymatic degradation expands the tissue damage. Early intervention in this phase could therefore be an effective therapeutic approach. To model the injury onset and recovery process, and study the consequent inflammatory environment in the heart, we are first establishing a porcine ischemia-reperfusion injury animal model. We will be utilizing endovascular techniques on Finnish landrace swine to induce an acute myocardial infarction, followed by reperfusion. The porcine heart serves as an appropriate and translationally convenient modeling system for cardiac research due to its close resemblance to

the human heart in terms of physiology and coronary anatomy. In our upcoming work will perform flow cytometry during the three-day inflammatory peak to characterize and quantify immune cells, and additionally assess heart function parameters and infarction severity at both the three-day time point and 1-month end point with MRI and echocardiography.

P631

Gene surgery as a potential treatment option for Nephropathic Cystinosis in vitro

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Nephropathic cystinosis is a rare monogenetic kidney disease caused by mutations in the lysosomal transporter cystinosin (*CTNS*) that, to date, cannot be cured. The hallmark of this disease is lysosomal accumulation of cystine and decline in kidney proximal tubular function leading to kidney failure early in life. In this project, we aimed to develop a novel gene repair strategy for the most predominant disease-causing variant, a 57Kb deletion in *CTNS*, using CRISPR/Cas9 Homology-Independent Targeted Integration (HITI). To model the disease phenotype, we developed two conditionally immortalized proximal tubule epithelial cell (ciPTEC) lines. A $CTNS^{Patient}$ ciPTEC line was obtained from a cystinosis patient harboring the 57Kb deletion and an isogenic $CTNS^{-/-}$ ciPTEC line was generated from $CTNS^{WT}$ ciPTEC using CRISPR/Cas9. A novel, non-viral peptide-mediated delivery approach was used to introduce the Cas9-guideRNA ribonucleoprotein (RNP) complex and various repair templates. To optimize delivery, we created a repair construct containing only mCherry (1.7 kb), only *CTNS* Superexon (1.7 kb) and a repair construct containing both mCherry and the *CTNS* Superexon (mCherry_Superexon, 3.2kb). After introducing the various repair constructs, we evaluated the percentage of mCherry positive cells using flow cytometry and found that the smaller mCherry construct (1.7 Kb) was able to achieve a higher repair efficiency (up to 63 % of cells) compared to the *CTNS*-mCherry construct (3.2 Kb) with a 16% integration efficiency. Clonal expansion of individual cells showed restoration of lysosomal cystine levels in 60%-70% of the clones when compared to the $CTNS^{WT}$ cells ($p<0.01$-0.0001), confirming that in most of the cells the *CTNS* function was restored, which was accompanied by improved mitochondrial bioenergetics in $CTNS^{Patient}$ cells. In conclusion, CRISPR/Cas9 HITI can be used to precisely insert repair templates into the genome, resulting in a functional cystinosin transporter restoration, and a reversal of the cystinotic disease phenotype in proximal tubular cells. This gene repair system may offer a potential curative therapy for cystinosis, as well as a system for the *in vitro* restoration of several other genes involved in monogenic diseases.

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Discovery and engineering of hypercompact epigenetic modulators for durable activation of therapeutic gene targets

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The ability to programmably modulate transcription in human cells enables an emerging therapeutic modality for epigenetic control over disease-associated gene expression without the need for changes to the genomic sequence. Recent CRISPR-based strategies using RNA-guided, nuclease-dead Cas effector (dCas) for recruitment of epigenetic modulators to target loci have shown high specificity and efficacy, but clinical applications are limited by large cargo sizes and temporally transient on-target activity windows of existing functional domains, particularly in cases where durable gene activation is desired. By using an in-house engineered CRISPR-Cas effector (dCasOnyx) and shortened gRNA scaffolds, we achieve a recruitment platform of sufficient compactness to enable greater cargo capacity (>2kb) and thereby flexibility in epigenetic modulator fusions. Here, we expand the epigenetic control toolkit by high-throughput discovery of novel transcriptional modulator domains from diverse proteomes and characterize their utilities in terms of potency, context-independent robustness, and durability of activity across a multitude of target genes. We report the discovery of hundreds of compact peptides (85aa) achieving potent gene activation or suppression, and describe a predictive biochemical rubric for their accelerated discovery from unannotated proteomes. Leveraging this discovery pipeline, we further describe a novel few-shot machine learning approach for de novo protein design in silico, yielding improved activation hit rates of 23% versus the <1% of traditional proteome tiling-based screens. Notably, we identified a minimal core activator domain (vCD; 32aa) capable of both high potency and exceptional durability, leading to mitotically stable target gene activation through dozens of cell divisions after a single delivery via dCas-mediated recruitment. Semi-rational engineering of the vCD resulted in dozens of hypercompact activator variants (64-100aa) with potency profiles similar to larger multi-domain industry benchmarks, while displaying the novel feature of durability absent in these benchmark tools and occupying only ~12-20% of their protein-coding cargo size. Mechanistic studies revealed that selective inhibition of CBP/P300-associated BRD bromodomains abrogated the activation durability of vCD, implicating a histone acetylation-mediated epigenetic memory mechanism for transmission of mitotically stable gene activation. Finally, we demonstrate potent activation by vCD-based modulators of haploinsufficiency disease-modifying genes in relevant cell models, paving a path toward in vivo proof-of-concept studies. Together, our discovery pipeline provides a predictive rubric for the systematic discovery and engineering of hypercompact activators, yielding superior potency and kinetics profiles that broadly expand the potential therapeutic landscape.

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Design and characterization of compact and precise Cas molecules for treating diseases in patients

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Treating intractable diseases requires the development of genome engineering molecules that deliver highly controllable outcomes and have broad targeting scopes. By combining the precision of CRISPR-Cas enzymes and novel genome modulators, Epic Bio is refining epigenetic editing at the forefront of gene therapy to control gene expression and mitigate and reverse diseases that are beyond the reach of genome editing.

Our engineering efforts have led to the discovery of highly compact nuclease-deficient dCas enzymes with broad targeting capabilities, thus enabling efficient delivery by AAV due to their small sizes (one third the size of Cas9), and avoiding double-strand breaks or permanent edits. All clinical applications of Cas-based genome engineering are constrained by the requirements for protospacer-adjacent motifs (PAM) in the target DNA. Importantly, our novel dCas molecules display flexible PAM requirements and are easily programmed to target therapeutic genes.

Here we present two novel cellular assays for comprehensive PAM characterization that faithfully report the PAM requirements of diverse dCas proteins in human cells. These assays enable accurate detection of greatly expanded PAM profiles for our lead dCas effectors (dCasOnyx, dCasRuby, dCasTopaz), enabling the efficient targeting of disease-causing genes. These assays enable ongoing engineering and characterization of our novel dCas in relevant genomic contexts to facilitate their translation to therapeutics.

Altogether, we present our work to optimize compact and precise Cas molecules at the core of our epigenetic editing platform and demonstrate their broad utility, representing a major advancement towards treating intractable diseases in patients.

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CRISPRon-ABE: deep learning CRISPR adenine base editing design from data generation

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CRISPR base editing holds the promise to overcome the DNA repair challenges observed in DNA cleavage-based gene-editing. In for example, the Adenine Base Editor (ABE) all As in an editing window of ~8 nt inside the DNA target can with different frequencies be changed to Gs. The design task is then to predict the frequency of the A to G outcome and to predict the overall guide RNA (gRNA) editing efficiency. To make base editing applicable, efficient and accurate design tools are needed. Computational design models are emerging, but are still in a relatively early phase and data to make them from are limited. We have addressed this through generation of new data using

our SURRO-seq technology to measure the efficiency and outcome frequencies on ~11,500 gRNAs for which the CRISPR/Cas9 activities have previously been determined. To seek model advancements, we combine our generated data with recently published base-editing datasets, and construct a deep learning-based ABE efficiency predictor, CRISPRon-ABE, trained and evaluated on non-overlapping subsets of the in total ~18,000 gRNAs. Rather than fusing the different datasets by their efficiencies we constructed the model so that it is simultaneously trained on the different datasets using their respective efficiency scales. The fused model significantly improves the performance on the independent test set compared with the training by any one single dataset. Outcome frequencies should be considered in the context of gRNA editing efficiency, and therefore we simultaneous evaluate gRNA efficiency and outcome frequency using a K-dimensional extended Pearson correlation coefficient RK (K=2) for the evaluation. Overall, the performance benefits from the dataset fusion; even for the DeepABE testset used for the current benchmark method, we obtain R2=0.82 clearly exceeding R2=0.72 by DeepABE, as well as R2=0.63 by BE-HIVE and R2=0.37 by BE-DICT. We find that the improvement is in part due to the increased number of gRNAs and outcomes, and in part due to a better model architecture.

P635

Efficient and precise RNA-templated DNA repair using TevCas9 gene editors

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The programmable, efficient and precise replacement of genomic sequences in cells using gene editing technologies has enabled rapid advances in research and as an approach to treat genetic diseases. RNA-programmable gene editing techniques such as CRISPR-Cas9 mediated homology-directed repair (HDR), based editing and PRIME editing have been developed to insert, delete or replace sequences at a target site while minimizing unwanted editing byproducts. However, limitations may prevent the use of these technologies for certain target sites and cells due to low frequency of the HDR pathway, unwanted bystander indels, target site limitations such as nearby bystander bases for base editing or variable activity of the gene editors in different cell types. Additionally, the coding size of many gene editors and the challenge of co-packaging RNA and donor DNA in non-viral vectors are additional barriers to delivering gene editors for *in vivo* therapeutic gene editing. Delivery of regulatory sequences, gene editor, guide RNA(s) and repair sequences as one construct is desirable, particularly for expression in a single adeno-associated viral vector (AAV).

Here, we present proof-of-concept using an RNA-guided two-site TevCas9 gene editor for the efficient and precise replacement of sequences using RNA-templated DNA repair in mammalian cells in the absence of an exogenous reverse transcriptase. TevCas9 gene editors are fusions of the GIY-YIG family I-TevI nuclease and linker domains to RNA-programmable nuclease domains, which in this study is *Staphylococcus aureus* Cas9. Using a single AAV encoding TevCas9, the guide RNA and repair sequence in one construct (“all-in-one”), we show accurate target site replacement at the AASV1 genomic safe harbour site by deep sequencing with virtually no detectable editing byproducts (indels or inaccurate repair template insertions). Repair is also detected at multiple sites and in a variety of cell types using an “all-in-one” plasmid DNA construct, or with an “all-in-one” messenger RNA, or with purified TevCas9 protein complexed with a single guide RNA-repair template. Lastly, we propose a mechanism whereby polymerase theta (θ) is recruited to the target site to reverse transcribe the RNA template to mediate DNA repair.

P636

Adenine base editor-mediated correction of three prevalent and severe β-thalassemia mutations

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β-thalassemia is a highly prevalent monogenic recessive disease caused by mutations affecting the synthesis of the adult hemoglobin β-chains. Point mutations in the β-globin gene (*HBB*) locus reduce (β^+) or abolish (β^0) the production of β-globin chains. The imbalance between α- and β-globin production leads to the precipitation of uncoupled α-globins, which causes erythroid cell death, ineffective erythropoiesis and severe anemia. The most severe form of the disease, β-thalassemia major, is typically associated with a β^0/β^0 genotype. Transplantation of autologous, genetically modified hematopoietic stem/progenitor cells (HSPCs) is an attractive therapeutic option. However, current gene therapy strategies based on the use of lentiviral vectors or CRISPR/Cas9 nuclease are not equally effective in all the patients and/or raise safety concerns. Base editing, a new CRISPR/Cas9 derived genome editing tool, allows the effective introduction of point mutations (C>T by cytidine base editors or CBEs; A>G by adenine base editors or ABEs) at precise locations within the genome.

The two β^0 mutations CD39 (CAG>TAG) and IVS2-1 (G>A) and the β^+ mutation IVS1-110 (G>A) are among the most common and severe β-thalassemia mutations in the Mediterranean area and Middle East. Here, we exploited the capacity of ABEs in combination with specific single guide RNAs (gRNAs) to convert A>G and correct these mutations in HSPCs from β-thalassemia patients.

First, we screened different combinations of ABEs/gRNAs in T cells from β-thalassemia patients harboring these mutations and selected the best performing combination for each strategy. We then tested selected ABEs/gRNAs combinations in HSPCs from β-thalassemia patients and achieved correction efficiencies of up to ~90%. Control and edited β-thalassemic HSPCs were differentiated towards the erythroid lineage to evaluate globin and hemoglobin production. Red blood cells derived from edited HSPCs exhibited high β-globin levels and improvement of the α/non-α globin ratios. The delayed erythroid differentiation typically observed in β-thalassemic cell cultures was corrected by our treatment.

Finally, corrected HSPCs were transplanted in immunodeficient mice to evaluate the engraftment and differentiation capability of edited HSCs. We detected good frequencies of human cells with a mutation correction rate of up to 75% in the bone marrow of transplanted mice. Importantly, correction of the β-thalassemic phenotype was observed in vivo, as indicated by the high β-globin levels and the increased enucleation rate observed in erythroid cells. Moreover, serial xenotransplantation experiments showed base editing in long-term HSCs.

Overall, our study provides in vitro and in vivo proof of efficacy of a base editing approach to treat patients with prevalent and severe β-thalassemia mutations. The clinical development of our approach will require the establishment of a large-scale electroporation protocol with clinical-grade reagents and toxicology studies to demonstrate the safety of the base-edited drug product.

P637

Targeted base editing models hematopoiesis and pathophysiology of VEXAS syndrome

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VEXAS syndrome is a recently described, adult-onset, X-linked, life-threatening, autoinflammatory and hematological disease with high prevalence (~1:4,000 males over 50 years). It is caused by somatic mutations in the *UBA1* gene in hematopoietic stem/progenitor cells (HSPCs) and is characterized by the formation of vacuoles in myeloid and erythroid progenitors. About 50% VEXAS patients develop hematologic malignancies, including myelodysplastic syndrome, contributing to the poor prognosis. However, the pathological mechanisms and impact of VEXAS syndrome on human hematopoiesis remain elusive and the lack of disease models limits the understanding about the disease development as well as the preclinical testing of effective pharmacological interventions. Here, we develop *in vitro* and *in vivo* models of VEXAS syndrome by targeted base editing strategies and generate multi-omics data on our cohort of VEXAS patients to cross-validate them. We designed single guide RNAs able to install VEXAS-causing mutations in the *UBA1* gene by adenine base editors. Editing efficiency approached 90% in wild-type myeloid cell lines, primary human T cells and HSPCs. While *UBA1*-mutant T cells were counterselected over time in culture, myeloid ones were enriched. Colony-forming unit assay of *UBA1*-edited HSPCs revealed an exclusive myeloid output *in vitro* and a significant loss of the erythroid HSPC differentiation potential compared to mock-edited controls. When transplanting *UBA1*-mutant HSPCs in immunodeficient mice, we observed poor human hematopoietic reconstitution. Reduced graft size was due to a dramatic shrinkage (~100-fold) of the lymphoid compartment, while NK and myeloid outputs where partially preserved. Concordantly, analyses of the long-term graft in the bone marrow of hematochimeric mice highlighted high content of CD34+ HSPCs, which were predominantly myeloid-biased and presented abnormal physical parameters, likely due to vacuolization. Targeted sequencing of the *UBA1* allele showed >80% VEXAS mutation in myeloid cells and HSPCs, but predominance of wild-type alleles within the lymphoid compartment. Strikingly, the distribution of *UBA1*-mutant cells across lineages in the humanized model mirrored that of VEXAS patients' cohort, where the *UBA1*-mutant cells accounted for 90% of the total myeloid cells and HSPCs and for less than 5% of the lymphoid lineages, in accordance with published reports. Multiparametric immunophenotypic analyses showed unbalanced composition of the HSPC compartment in patients' bone marrow, with lower abundance of stem cells, multipotent and lymphoid progenitors and higher abundance of myeloid progenitors compared to age-matched healthy individuals. This was paralleled by a significant increase of circulating myeloid-biased HSPCs, and immature myeloid cells. Transcriptomic analysis of patients' monocytes showed sustained VEXAS-driven inflammatory signatures and glycolysis upregulation, which were confirmed by metabolomic

analyses. Single-cell transcriptomic on the hematopoietic system of VEXAS patients and on the humanized model are ongoing. Competitive transplantation and clonal tracking experiments in the mouse model will ultimately shed light on the dynamics and selective advantage of *UBA1*-mutant HSPCs. Overall, our data show that base editing-mediated insertion of somatic mutations causing VEXAS syndrome in wild-type cells allows to phenocopy most features of patients' hematopoietic system. We envisage that these models will support the dissection of mechanistic bases underlying VEXAS pathogenesis and enable preclinical testing and validation of novel therapies.

P638

Oncogenic Extrachromosomal DNA Targeting Using Autophagy and Immune Activator

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Metastasis account for the great majority of cancer-associated deaths. Intra-tumoral heterogeneity contributes to cancer drug resistance, so the elucidation of underlying mechanisms is very important for controlling the growth and spread of primary tumors. Extrachromosomal DNA (ecDNA) was early described as "double minute" and recent studies unveiled that ecDNA is primary source of oncogene amplification in tumor cell. Integrating ultrastructural imaging, long-range optical mapping and computational analysis of whole-genome sequencing revealed ecDNA contains highly accessible chromatin where ecDNA drives massive oncogene expression. ecDNA was found in nearly half of human cancers but it was almost never found in normal cells, which shows that ecDNA is tumor-specific. Our data also showed there are prominent ecDNA accumulation in glioblastoma multiforme (GBM) cell line resistant to radiation therapy, chemotherapy and photodynamic therapy when compared with parental cells. Both TP53 and Ras play a crucial role in autophagy initiation and cells deficient in autophagy exhibit chromosome instability associated with a higher incidence of aneuploidy. We have demonstrated that knockout of autophagy regulatory gene, ATG5, in Ras-transform MEF cell possessed increased ecDNA. Moreover, knockout of ATG5 gene of SW480 cell with intrinsic TP53 mutation also acquired a prevalent ecDNA distribution. These data significant enforced the potential of autophagy activation in ecDNA elimination and tumorigenicity regulation. We revealed autophagy activating reagent and DNA repairing inhibitor exerted a striking cytotoxicity in Temozolomide- and Taxol-resistant GBM cells of which ecDNA and spheroid formation was remarkably repressed. Furthermore, targeting CD155/TIGIT pathway holds promise for developing NK cell-based immunotherapies of GBM, we also attested that an ep4 antagonist significantly depressed CD155 expression of Taxol-resistant GBM cells. Collectively, combination of autophagy activation or DNA repairing inhibitor with immune activator provide beneficial insights on conquering the chemotherapy resistance and serve as a novel therapeutic regimen for GBM treatment.

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Cas12 DNA editing restores dystrophin expression and muscle function in mouse model of Duchenne muscular dystrophy and demonstrates high editing efficiency in NHPs

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Duchenne muscular dystrophy (DMD) is a fatal X-linked neuromuscular degenerative disease affecting the majority of muscle groups throughout the body of 1 in 3,500 to 5,000 newborn boys worldwide. It is a childhood-onset monogenic disease caused by loss-of-function mutations in the *DMD* gene encoding the dystrophin protein which is critical for maintaining muscle integrity during contraction. In its absence, skeletal muscle fibers experience repeated bouts of necrosis and regeneration, eventually resulting in loss of muscle mass and function including muscle membrane fragility, myocyte necrosis, myocardial fibrosis, and inflammatory infiltration, which together with cardiomyopathy reduces life expectancy. Currently, there is no curative therapy available for this devastating disease. Due to the huge size of the *DMD* gene, there are few ongoing clinical trials aim to restore muscle integrity and function following micro-dystrophin (μ-Dys) gene replacement using adeno-associated viral vectors (AAVs). While gene replacement therapies hold great promise for ameliorating DMD pathology, the utilization of a μ-Dys gene expressing versions of truncated dystrophin, as necessitated by the limited carrying capacity of AAVs, suggesting that some aspects of dystrophin functionality are sacrificed as discussed by the US FDA during recent advisory committee for an AAVrh74-*DMD* gene replacement product. Therefore, there is a significant unmet medical need to explore innovative and effective therapeutic approaches to correct genetic mutations and improve motor functions. Among thousands of DMD-causing mutations, corrections of the skipping and reframing of exon 51 are estimated to therapeutically benefit at least 14% of all DMD cases worldwide. Here, we generated a novel humanized DMD mouse model harboring human *DMD* exon51, which exhibited highly similar phenotypes in patients with DMD. Then, we developed an efficient single-cut gene-editing method using a compact high-fidelity Cas12i variant with high editing activity and specificity, hfCas12Max, packaged in a single AAV to restore exon51 open reading frame in *DMD*. Our results showed that hfCas12Max efficiently restored 50% (*P<0.01*), 80% (*P<0.01*), and 70% (*P<0.01*) of dystrophin expression in heart, diaphragm as well as tibialis anterior and ameliorated pathologic hallmarks of DMD, including histopathology and muscle function (improved grip strength by 50%) in this mouse model. Furthermore, we also validated the gene editing efficiency of *DMD* in wild-type (WT) non-human primates (NHPs) via systemic administration of AAV-hfCas12Max-gRNA. Both skeletal and cardiac muscle demonstrated robust editing efficiency in mean DNA levels (10%, ranging 3-21%) and mean RNA levels (22%, ranging10-40%). In addition, no *in vivo* toxicities were observed, including nerve, liver, and kidney injury, in the humanized DMD mice and WT NHPs. Unlike antisense-oligonucleotide-based exon skipping therapy administering throughout a patient's lifetime or AAV gene replacement therapy expressing micro-dystrophins for partial dystrophin functionality, our innovative CRISPR-based hfCas12Max-mediated single-cut approach modifying genomic DNA offers a promising, long-lasting, and "one-and-done" new treatment modality that targets the underlying cause of DMD.

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Efficient repression of HBV by targeted epigenetic editing

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Hepatitis B virus (HBV) is a hepatotropic DNA virus that causes life-long infections associated with an increased risk of fibrosis, cirrhosis, and hepatocellular carcinoma. HBV persists in the hepatocytes of chronically infected patients as episomal nuclear DNA, known as covalently closed circular DNA (cccDNA), which acts as a highly stable reservoir for viral replication. Additionally, integrated viral DNA (intDNA) produces hepatitis B surface antigen (HBsAg) which suppresses the host immune system and inhibits viral clearance. Whereas current pharmacological interventions profoundly inhibit viral replication and frequently result in a functional cure, their discontinuation often leads to HBV rebound, a consequence of the persistence of - an expression from - cccDNA and intDNA. Here, we present epigenetic editing (epi-editing) as a potential therapeutic approach for chronic hepatitis B. Epi-editing exploits engineered proteins comprising a programmable DNA binding domain, either catalytically deactivated Cas9 (dCas9), ZFPs, or TALEs, fused to epigenetic effector domains. These proteins can target specific DNA sequences to introduce mitotically heritable repressive marks, such as methylation at CpG dinucleotides. Compared to alternative gene editing approaches, epi-editing offers the advantage of achieving permanent transcription repression without the genotoxic risks associated with the induction of DNA breaks. We initially design a library of hundreds of individual guide RNAs (gRNAs) targeting potential regulatory regions (i.e., CpG islands, promoters, and enhancers) throughout the entire HBV genome. These gRNAs were prioritized according to conservation across HBV genotypes. To identify the best-performing gRNAs, we performed an arrayed screen in an HBV-infected HepG2 cell line that overexpresses the cognate viral entry receptor, NTCP. To transiently deliver mRNAs encoding for the dCas9-based epi-editors and gRNAs, we exploited lipid nanoparticles (LNPs). In these studies, we successfully identified gRNAs that exhibited efficient HBV repression (>70% reduction of HBsAg and HBeAg production). The efficacy of the high-performing epi-editors was confirmed in HBV-infected cultured primary human hepatocytes, where we observed up to 80% reduction in key viral antigens. Finally, whole transcriptomic profiling of treated cells revealed that the selected epi-editors were highly specific. In summary, we provided *in vitro* proof-of-principle of the effective and specific silencing of HBV through targeted epigenetic editing.

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Unique features of ARCUS nucleases enable high efficiency, targeted gene insertion in vivo

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Delivery of therapeutically relevant genes *in vivo* via adeno associated virus (AAV) gene therapy has shown promising clinical results in numerous indications. However, expression from episomal AAV is hampered by vector dilution in tissues that experience cellular turnover, potentially limiting the therapeutic durability. Nuclease-driven, targeted gene insertion into the genome is an appealing therapeutic approach to overcome these challenges. By inserting a wild-type version of a mutated DNA sequence into the genome of affected cells, functional protein can be produced, and edits can be permanently retained through cell division. This approach can potentially lead to longer durability and more consistent therapeutic effect. However, achieving therapeutically meaningful levels of gene insertion has been challenging for many gene editing nuclease platforms, especially in non-dividing cells. Here, we describe the use of ARCUS nucleases for targeted gene insertion across a variety of in vitro and in vivo models. Unique amongst gene editing technologies, ARCUS nucleases create double strand DNA (dsDNA) breaks with 4 base pair, 3' overhangs. To determine if the 3' overhangs were important for gene insertion, we fused an ARCUS nuclease to a 3' exonuclease protein and evaluated the impact on insertion efficiency. We found that removal of the 3' overhangs resulted in ~7.5x lower gene insertion efficiency in cultured cells. To further confirm the unique advantage of the ARCUS overhangs to promote high efficiency gene insertion, we dosed newborn and infant nonhuman primates (NHPs) with two AAVs containing the ARCUS nuclease and gene insertion construct. Using two different gene insertion constructs, we achieved 11.9-21.2% gene insertion. To determine if similar rates of editing could be achieved using lipid nanoparticle (LNP) delivery of ARCUS nucleases in older NHPs, we co-administered AAV containing the gene insertion construct and LNPs formulated with ARCUS mRNA and then measured targeted gene insertion in liver. We observed up to 45% gene insertion in bulk liver tissues which was maintained at 1- and 3-months post AAV/LNP administration. Using this same nuclease and delivery strategy, we also observed similar levels of gene insertion in non-dividing, primary human hepatocytes, suggesting good translatability across models and species. Together, these studies suggest a critical role of the 3' overhangs generated by ARCUS nucleases for achieving high levels of gene insertion in vivo and highlight unique capabilities of the ARCUS gene editing platform for therapeutic targeted gene insertion.

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Targeted knock-in of a promoter-less *NCF1* cDNA at the endogenous *NCF2* corrects p47^{phox}-deficient chronic granulomatous disease in a myeloid fashion

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The 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, CGD patients often suffer from life-threatening bacterial and fungal infections, as well as hyperinflammation. A potential curative treatment for CGD is the transplantation of genetically modified autologous hematopoietic stem and progenitor cells (HSPCs). Here, we explore gene editing in HSPCs as an alternative treatment to allogeneic hematopoietic stem cell transplantation.

We developed a targeted knock-in strategy, using CRISPR-Cas9 by insertion of a construct that consists of a 2A oligopeptide and the *NCF1* cDNA (2A-*NCF1*) into the 3' end of the *NCF2* gene. *NCF2* encodes for p67^{phox}, another NADPH oxidase subunit that is highly expressed in myeloid cells. The knock-in relies on the electroporation of CRISPR-Cas9 ribonucleoprotein and delivery of the 2A-*NCF1* template by integration-deficient lentiviral vectors (IDLV), or by adeno-associated viral vectors (AAV). The use of a promoter-less vector is intended to ensure physiological expression pattern of p47^{phox} under the regulation of the *NCF2* promoter and to reduce the risk of insertional activation in case of off-target integration events.

This strategy corrected the disease phenotype in a human p47^{phox}-deficient myeloid cell line (PLB-985 *NCF1* ΔGT) and in p47^{phox}-deficient mouse HSPCs: knock-in of the *NCF1* cDNA at the *NCF2* locus successfully restored p47^{phox} expression upon myeloid differentiation in 33.5 ± 5.3% (AAV template) and 32.2 ± 0.7% (IDLV template) of edited PLB-985 *NCF1* ΔGT cells, and led to reconstitution of NADPH oxidase function in 18.9 ± 4.6% and 34.0 ± 5.3% of edited cells, respectively. In healthy human CD34+ cells, knock-in efficiencies of 25.9 ± 4.7% were achieved. In edited p47^{phox}-deficient mouse HSPCs, we observed 41.6 ± 16.6% of p47^{phox}-expressing cells and restored NADPH oxidase function in 25.0 ± 7.3% of edited cells. Moreover, we confirmed that transgenic p47^{phox} expression followed myeloid differentiation and was under the control of *NCF2* promoter.

We then investigated the impact of potential fusion proteins resulting from inefficient 2A activity. Fusion proteins of p67^{phox}-2A-p47^{phox} were detected in <1% of edited bulk cultures, with no related effect observed on the NADPH oxidase function. We also characterized the incidence of off-target vector integration events using droplet digital PCR by measuring copy numbers of viral-derived elements.

In conclusion, we developed a novel gene therapy strategy for p47-CGD linking the *NCF1* cDNA to the *NCF2* locus, ensuring spatiotemporal transgene expression that is limited to myeloid cells.

P643

BEAM-302 decreases hepatic aggregates of mutant AAT and increases circulating functional AAT in rodent models of Alpha-1 Antitrypsin Deficiency

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Alpha-1 antitrypsin deficiency (AATD) is an inherited disorder caused by mutations in the *SERPINA1* gene that encodes an anti-protease, Alpha-1 Antitrypsin (AAT). The PiZ mutation, a G-to-A polymorphism in the *SERPINA1* gene, is the most common mutation associated with severe AATD. Mutant AAT (Z-AAT) misfolds and forms aggregates that are proteotoxic to the liver. The inefficient secretion of Z-AAT leads to a deficiency of circulating AAT that can result in lung damage due to unopposed elastase activity. The PiZ mutation is an ideal target for correction to wildtype (PiM) by an adenine base editor (ABE) which converts an A to G in genomic DNA. BEAM-302 is a lipid nanoparticle (LNP) formulation of an mRNA encoding an ABE and a guide RNA (gRNA) targeting correction of the PiZ mutation. The pharmacological activity of BEAM-302 was characterized in the NSG-PiZ mouse model of AATD that carries multiple copies of the human PiZ transgene and demonstrates liver aggregates of Z-AAT. BEAM-302 was also evaluated in a novel rat model (PiZ rat) with a 1:1 replacement of rat AAT with human Z-AAT. In both models, a single, systemic administration of BEAM-302 induced dose-dependent rates of editing in liver. Base editing was associated with decreased Z-AAT aggregates in NSG-PiZ mouse livers and corresponding increases in circulating functional AAT in both models. Furthermore, repeat administration of BEAM-302 resulted in further increases in editing rates. Taken together these data support the hypothesis that base-editing by BEAM-302 has the potential to mitigate both the liver and lung pathology of AATD.

P644

Prime editing functionally corrects Cystic Fibrosis causing mutations in patient-derived organoids and airway epithelia

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Cystic Fibrosis (CF) is one of Europe's most common monogenic, lethal diseases and has been estimated to affect >160.000 people world-wide. CF is caused by recessive mutations in the *CFTR* gene encoding the CFTR protein, an essential chloride and bicarbonate channel in the apical membrane of secretory epithelia. Absence of or defects in CFTR lead to a disrupted salt-water balance in multiple organs including lungs, pancreas and intestines. Being a severe and monogenic disease, gene therapy for CF has been pursued since the early 90's and focused on a gene addition approach. Gene correction on the other hand, would allow to restore mutations on

the patient chromosomes, thereby preserving endogenous gene expression, regulation and possibly provide a permanent cure. We hypothesized that prime editing (PE), one of the most recent and powerful CRISPR-derived technologies, could be leveraged to permanently and precisely correct drug-refractory mutations, such as L227R (c.680 T>G) and N1303K (c.3909 C>G).

To obtain optimal PE efficiency, we screened over >20 prime editing guide RNA (pegRNA) and nicking guide RNA (ngRNA) combinations for both mutations in *CFTR*-cDNA engineered HEK293T cells. To further enhance editing efficiencies, we applied SotA PE systems including mismatch repair modulation strategies, 3' protected epegRNAs and optimised PE enzymes. In HEK, correction of the *CFTR*-cDNA resulted in restored CFTR glycosylation (band C on Western blot), plasma membrane localisation (immunocytochemistry followed by confocal microscopy or flow cytometry) and CFTR function (halide-sensitive YFP quenching assay).

To validate our approach on the endogenous *CFTR* gene and in more translational models, we delivered the developed PE approaches to patient-derived rectal organoids of five different patients, human nasal epithelial cells from two different patients and engineered 16HBE-YFP cells via lentiviral vectors (LV). In each model genomic correction of the endogenous *CFTR* locus was associated with clear functional correction (up to 80% in patient-derived organoids and 30% in airway epithelial cells). To facilitate future gene editing research and screening, we built a machine learning analysis tool (DETECTOR) capable of fast and sensitive quantification of functional correction in primary organoids. With the sensitive analysis enabled by DETECTOR, we were able to accurately dissect the effect of each of the PE components/enhancements on editing outcome. Use of ngRNAs (PE3b approach), 3' protected pegRNAs (tevopreQ1-epegRNA) and modulation of mismatch repair (PE4 or P5 strategy) for instance delivered significant increases in functional correction.

To investigate the safety-profile of the selected pegRNAs and ngRNAs, we employed *in silico* prediction, as well as experimental discovery (GUIDE-Seq) to identify sites prone to SpCas9 off-target editing. Deep sequencing on these sites revealed no significant off-target editing in cells treated with the developed PE approaches. Interestingly, these results were also confirmed in patient-derived organoids subjected to LV-mediated and prolonged (14 days) expression of PE, pegRNA and ngRNA. These data further confirm on the reported and remarkable safety profile of PE.

In summary, our results show that L227R and N1303K, both severe CF-causing mutations, can be safely corrected by PE. Ongoing work focuses on tailoring a translational delivery vehicle for the developed gene editing strategies.

P645

Gene editing of HLA-class I and class-II facilitates successful allogeneic 'off-the-shelf' regulatory T cell therapy

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Adoptive transfer of regulatory T cells (Treg) holds significant promise in the treatment of pathological immune responses, particularly in cases of autoimmunity, graft-versus-host disease (GvHD), and transplant rejection. Currently, Treg cells are predominantly derived from each patient individually, followed by ex vivo expansion and re-infusion into the same patient. However, autologous approaches are impeded by patient-specific variabilities, manufacturing challenges, and time-consuming expansion protocols. The individualized therapy results in high costs and hinders the application in acute situations. Allogeneic Treg cells from healthy donors have the potential to overcome these obstacles, while allo-specific host immune responses targeting mismatched HLA pose a new one. Due to their inherent immunosuppressive capacity, it remains unclear to which extent allogeneic Treg cells can prevent allo-rejection by the host's immune system. Therefore, we compared the suppressive capacity of allogeneic and autologous Tregs in different models and devised gene editing strategies to improve their persistence and efficacy in immunocompetent hosts.

While allogeneic and autologous Treg cells exhibited similar suppressive functions in vitro, the ability of allogeneic Treg cells to prevent skin allograft rejection in vivo was significantly diminished. In immunodeficient mice engrafted with peripheral blood mononuclear cells (PBMCs), allogeneic Treg cell numbers rapidly declined compared to autologous Treg cells. Conversely, when CD8+ T cells were depleted from the PBMCs, allogeneic Treg cell numbers remained comparable to autologous Treg cells. This underscores the influence of CD8+ T cells in the allo-response directed towards Treg cells. Consequently, we investigated whether genetic manipulation of HLA expression could improve the survival of allogeneic Treg cells. Through CRISPR-Cas9 gene knock out (KO) of *B2M* and *CIITA* we achieved highly efficient disruption of HLA class I and class II expression, respectively. To assess the ability of such HLA-engineered Treg cells to resist allo-response, we conducted in vitro co-cultures with allo-specific T cell lines comprising CD4+ and CD8+ T cells. Silencing both HLA class I and class II significantly enhanced protection against allo-specific T cell cytolysis, in comparison to *B2M*-single-edited or wild-type Treg cells. As Treg lacking HLA class I expression were susceptible to missing-self recognition and subsequent NK cell-mediated cytolysis, we introduced HLA-E into the *B2M* locus via non-viral knock-in (KI). Treg cells expressing the HLA-E-B2M fusion protein were partially protected from NK cell lysis in vitro. Finally, allogeneic Treg cells with HLA-E-*B2M* KI and *CIITA* KO demonstrated prolonged survival of skin grafts, similar to autologous Treg cells. In contrast, Treg with HLA-E-*B2M* KI, and intact mismatched HLA-class II expression, failed to confer comparable long-term protection.

These results indicate that silencing both HLA class I and class II is essential for the survival of allogeneic Treg cells in immune competent hosts. Combined with HLA-E KI, our gene editing strategy enabled allogeneic Treg cells to evade allo-specific T cell and NK cell recognition. Consequently, these cells persisted for a sufficient duration to establish a regulatory environment, providing a comparable level of allograft protection to autologous Treg cells. Our findings highlight

the potential of HLA-engineered 'off-the-shelf' Treg cells for the treatment of various inflammatory diseases.

P646

Intronic editing enables lineage specific expression of therapeutics relevant for HSPC gene therapy

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Safe harbor gene editing is commonly used to express therapeutics from hematopoietic stem and progenitor cells (HSPC) or their progeny. However, this approach generally relies on constitutive promoters that render expression of a transgene ubiquitously. We propose a promoter-less intron editing approach that relies on the cellular RNA splicing machinery to induce lineage-specific expression of a therapeutic transgene exclusively upon differentiation.

Our results show that TALEN®-mediated intron editing of CD4, CD20, and CD11B genes resulted in the lineage- specific expression of a reporter transgene in T-cell, B-cell, and myeloid cells, respectively, with negligible expression in HSPC or other cellular subsets *in vitro*. Importantly, we confirmed *in vitro* that our intron editing approach does not negatively affect the expression of the targeted endogenous gene targeted, using CD11B as a locus model.

CD11B-edited HSPCs injected in an immunodeficient mouse model resulted in myeloid specific transgene expression in bone marrow, blood and spleen. Additionally, among human cells reaching the mouse brain, 20.6% were edited, and a similar percentage (23%) was observed within the human microglial compartment.

When inserting the alpha-L-iduronidase (IDUA) therapeutic gene into the intron of CD11B in HPSC, *in vitro* myeloid differentiation led to a 10-fold supra-endogenous expression of IDUA, potentially enabling efficient cross-correction. *In vivo*, IDUA-edited cells reached blood, bone marrow, spleen, and brain at allelic editing rates of 14.9%, 9.9%, 12.3%, and 18.9% respectively.

We believe this intron editing approach could be disruptive in HSPC gene therapy and brain delivery of therapeutics.

P647

Novel and compact CRISPR nucleases for the allele-specific and mutation-independent treatment of autosomal dominant retinitis pigmentosa

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Autosomal dominant mutations are responsible for almost one third of all Retinitis Pigmentosa cases (adRP), a common form of severe retinal degeneration, with the majority of defects falling in the RHO gene, encoding the photopigment Rhodopsin. However, the high genetic heterogeneity of patients (>200 mutations) makes the development of a universal gene editing approach to treat adRP challenging.

We have developed a mutation-independent CRISPR-based strategy to selectively abolish the expression of the mutant toxic form of RHO, by specifically introducing a deletion in the mutated allele. Our strategy exploits a combination of two sgRNAs targeting respectively RHO intronic regions (bi-allelic cut) and a high-frequency single nucleotide polymorphism (SNP) within the RHO gene, which has to be present in heterozygosis in patients to allow allele phasing and discrimination. This strategy requires the ability to discriminate single nucleotide differences among genomic targets in order to selectively edit the desired SNP allele and was originally optimized using high-fidelity variants of SpCas9 we had previously engineered. However, technical limitations intrinsic to SpCas9 such as deliverability, due to its large size, and immune responses can limit the translatability of the approach *in vivo*.

We thus exploited our metagenomic platform to mine the human microbiome for novel CRISPR nucleases which can efficiently target the RHO gene and are characterized by compact size (<1100 aa) to improve retinal delivery using all-in-one AAV vectors. By leveraging on our in silico PAM prediction capabilities, we were able to identify several candidates to edit the SNP locus, which were then tested side-by-side and ranked for their allele specificity. Best performers were evaluated for their ability to consistently edit the target RHO intron and further optimizations of the top nuclease and sgRNAs were implemented to increase on-target activity and maximize allele discrimination. We then validated the genome-wide specificity of the approach and demonstrated the ability to selectively downregulate mutated RHO at the mRNA and protein level in minigene-expressing cells. Furthermore, thanks to the reduced protein size, the candidate nuclease and both sgRNAs were included into an optimized all-in-one AAV vector which was tested for efficient editing in target cells.

Our uniquely tailored approach based on the identification of compact, highly active and specific fully novel CRISPR nucleases from metagenomic samples offers a first-in-class mutation-independent and allele-specific targeting strategy for RHO-dependent adRP, which could be potentially extended to many other autosomal dominant conditions.

P649

CRISPR-Cas9 *RAG2*-SCID editing via Coding Sequence Replacement to Preserve Endogenous Gene Regulation and Locus Structure

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RAG2-SCID is a primary immunodeficiency caused by mutations in Recombination-activating gene 2 (RAG2) which is intimately involved in the process of lymphocyte maturation and

function. Ex-vivo gene editing of a patient's own hematopoietic stem and progenitor cells (HSPCs) using a combination of CRISPR-Cas9 and rAAV6 vectors could provide a safe therapeutic alternative to the only current treatment for RAG2-SCID, allogeneic hematopoietic stem cell transplantation (HSCT). To do so, we established transgene design for efficient homology directed repair (HDR) at the RAG2 genomic locus while maintaining endogenous regulatory and spatiotemporal elements. As a proof-of-concept single-allelic gene correction, we established a knock-in/knock-out (KI-KO) strategy in healthy donor (HD)-derived CD34+ HSPCs via multiplexed HDR. KI of our RAG2 correction donors that replaced the entire endogenous RAG2 coding sequence (CDS) to preserve endogenous gene regulation and locus architecture led to successful development from CD34+ HSPCs into CD3+TCRαβ+ and CD3+TCRγδ+ T cells and promoted the development of highly diverse TRB and TRG repertoires in an in-vitro T-cell differentiation (IVTD) platform. We present a method to correct the RAG2 gene while maintaining RAG2 expression regulation and locus structure and a workflow to determine the optimal configuration for CRISPR-Cas9/rAAV6 correction of genes with strict spatiotemporal gene regulation.

P650

Patterns of homology-independent targeted AAV integration at CRISPR/Cas9 induced-double-strand breaks

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AAV-mediated homology-independent targeted integration (AAV-HITI) by CRISPR/Cas9 at the highly transcribed Albumin locus provides sustained transgene expression following neonatal delivery in mice. We have shown therapeutic efficacy of AAV-HITI in a mouse model of Mucopolysaccharidosis type VI (MPSVI), a lysosomal storage disease due to deficiency of the secreted arylsulfatase B (ARSB). Liver-directed AAV-HITI in new-born MPS VI mice leads to supraphysyological and stable levels of serum-active ARSB which improve the mouse model phenotype. As AAV integration at CRISPR/Cas9 nuclease-induced DNA double-strand breaks (DSBs) raises safety concerns, we thoroughly evaluated on-target and off-target effects by CAST-Seq and next-generation sequencing (NGS). We show that AAV-HITI donor DNA is preferably integrated at the Albumin locus after CRISPR/Cas9 cleavage of the ITRs in the intended orientation. We additionally observed the integration of partial AAV genomes, including the ITRs, but no gross chromosomal rearrangements at the target locus. Furthermore, we neither detected insertion/deletion mutations nor AAV integration at predicted off-targets sites. Importantly, no evidence of hepatocellular carcinoma was observed within the 1-year follow-up after AAV-HITI. Overall, these data support the safety and efficacy of AAV-HITI directed to the Albumin locus and its further development for the treatment of conditions which involve the liver as target tissue and that require early intervention.

P651

CoCas9, a compact nuclease from the human microbiome for efficient and precise genome editing

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Genome editing tools based on CRISPR-Cas systems gave a tremendous impulse to the clinical development of advanced therapies. Yet, due to the complexity of genetic medicine, there is an urgent need of expanding the toolbox with smaller Cas proteins compatible with delivery tools and characterised by high on/off-target ratio on unrestricted genomic targets. To identify new CRISPR-Cas tools we interrogated a massively expanded microbiome repository, discovering, among a selected pool of small Cas9 proteins, a particularly active nuclease, CoCas9 (1004 amino acids). CoCas9 obtained remarkable editing levels (55% of indels) in human cells, showing similar efficiency to SpCas9 and improved precision. CoCas9 was also compared with other compact Cas9s exploited for single AAV delivery, achieving significantly higher efficiency than Nme2Cas9 and CjCas9, while showing similar activity to SaCas9 but generating far less off-target. A CoCas9-derived base editor (CoABE8e) showed high editing efficacy (up to 53% of A>G transition), with comparable activity to SaABE8e, Nme2ABE8, SauriABE8e and CjABE8e. Finally, we tested the compatibility of CoCas9 and CoABE8e with all-in-one AAV delivery, leading to a 30% of indels and 68% of base editing in transduced cells, respectively. To assess the in vivo activity of AAV-CoCas9, we targeted the human *RHO* gene in a knock-in mouse through subretinal injection, reaching up to 35.6% editing. Here we revealed a large reservoir of CRISPR-Cas systems from a massively expanded database from the human microbiome, out of which we selected and characterised CoCas9, a novel Cas9 able to efficiently and precisely edit the human genome.

P652

Targeting the *ATXN3* gene with CRISPR-Cas9 for the therapy of Machado-Joseph disease

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Machado-Joseph disease (MJD) is an autosomal dominantly inherited neurodegenerative disorder, caused by an over-repetition of the polyglutamine-codifying region in the ataxin-3 (*ATXN3*) gene. Expanded ATXN3 is prone to aggregate and disturbs diverse cellular systems, ultimately leading to cell dysfunction and death in specific neuronal populations. To date, no treatment able to reverse or stall MJD progression have been developed. Strategies based on the suppression of the deleterious gene products have demonstrated promising results in pre-clinical studies. Nonetheless, these strategies do not target the root cause of the disease, producing an incomplete and/or transient therapeutic effect in target cells or tissues. Recently gene-based therapeutics, including CRISPR-Cas9 systems for gene edition, have been successfully used to permanently inactivate disease-related genes, holding promise for the development of a definitive cure for inherited diseases.

Here, a panel of customized sequences were designed and constructed aiming at permanently suppressing the human *ATXN3* gene expression. Functional characterization of the designed system was initially performed in HEK293T cells, where its efficiency on gene disruption and consequent reduction of ATXN3 protein levels was demonstrated. Adeno-associated viral particles encoding these sequences were subsequently delivered in the striatum of a lentiviral-based mouse model of MJD by intracranial injection. Here, we observed a drastic reduction of the mutant protein, which resulted in decreased accumulation in ATXN3 aggregates in the striatum, thus preserving neuronal function. Importantly, the delivery of the engineered CRISPR-Cas9 system in the YAC-MJD84.2/84.2 MJD mouse model delayed the progression of the disease in comparison with non-treated littermates.

Overall, this work provides the first *in vivo* evidence of the efficacy of a CRISPR-Cas9-based approach to permanently inactivate the *ATXN3* gene, supporting its potential as a new therapeutic avenue in the context of MJD.

P653

ARCUS-mediated excision of the “hot spot” region of the human dystrophin gene results in functional improvement in a mouse model of Duchenne muscular dystrophy (DMD)

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Duchenne muscular dystrophy (DMD) is a genetic disorder associated with mutations in the dystrophin gene that prevent production of the dystrophin protein. Dystrophin stabilizes the cell membrane during muscle contraction to prevent damage, and the absence of intact dystrophin protein leads to inflammation, fibrosis, and progressive loss of muscle function and mass. Over time, children with DMD will develop problems walking and breathing, eventually leading to death in the second or third decade of life due to progressive cardiomyopathy and respiratory insufficiency. While a number of mutations in the dystrophin gene cause DMD, ~50% of patients contain mutations within a “hot-spot” region of the gene between exons 45 and 55. DMD occurs in 1 in 3,500 to 5,000 male births, and currently there are limited approved therapies available for patients. We have developed a gene-editing approach for the treatment of DMD, which could allow for a single administration of a drug with possible life-long benefits, and could be applicable

to the broad population of patients with mutations in the dystrophin hot-spot region. A pair of sequence-specific ARCUS nucleases that generate complementary 4 base pair, 3' overhangs at their target sites were engineered to excise exons 45-55, restore the reading frame of the dystrophin protein following perfect re-ligation of the gene, and generate a functionally competent variant of the dystrophin protein. Following administration of a single adeno-associated vector (AAV) encoding for the pair of ARCUS nucleases to a humanized DMD mouse model, we observed the edited dystrophin protein variant in multiple tissue types including heart, diaphragm, and skeletal muscle, with evidence of the edited dystrophin transcript in PAX7+ cells, a marker for muscle satellite cells. Furthermore, the maximum force output (MFO) of the gastrocnemius muscle in ARCUS-treated animals was significantly improved, reaching 86% of the MFO levels observed in non-diseased, control mice. This proof-of-concept study demonstrates therapeutic potential of an ARCUS gene editing approach for the treatment of DMD and supports ongoing development toward clinical candidate nomination.

P654

NHEJ inhibition increases homology-mediated AAV integration in hematopoietic cells

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Transplantation of autologous corrected Hematopoietic Stem and Progenitor Cells (HSPCs) is an attractive strategy for treating blood, immune and metabolic genetic disorders due to their self-renewal capacity and ability to differentiate into any blood cell type. One popular approach to edit HSPCs relies on gene editing tools such as CRISPR/Cas9 nucleases. The Cas9 induced double-stranded break (DSB) can be repaired via two main pathways: Non-Homologous End Joining (NHEJ), where generated free strands are re-ligated, or Homologous Directed Repair (HDR), which uses a template DNA for repair. By providing an external template of interest delivered by an adeno-associated virus (AAV), the latter can be hijacked to insert curative sequences at a chosen site. However, HDR activity is restricted solely to the S/G2 cell-cycle phases, considerably reducing the window of action of targeted integration. Moreover, the necessity for a high dose of AAV can trigger cytotoxicity, exacerbating these hurdles and ultimately lowering the efficiency of CRISPR-Cas9-based editing. NHEJ mechanism, instead, remains active throughout all phases of the cell cycle, competing with HDR for DSB reparation. To address these constraints, we tested a drug inhibiting a DNA-dependent protein kinase involved in NHEJ repair, aiming to favor HDR while simultaneously decreasing the AAV dose. We targeted the α-globin promoter, as we have previously shown it is a safe harbor site for DNA targeted integration in human HSPCs. In particular, we used Cas9-gRNA ribonucleoprotein complex alone or in conjunction with an AAV6 carrying a promoterless GFP and PGK-NGFR cassettes, flanked by homology arms to the genomic target site. Different concentrations of the NHEJ inhibitor (NHEJi) were tested on human HSPCs, using a low AAV6 dose, and the efficiency of DNA insertion was evaluated at different time points. GFP and NGFR signals were analyzed by flow cytometry. Treatment with NHEJi resulted in a ~2.5-fold increase in the GFP/NGFR + cells, going from 20% to 55% of the total population. This increase was confirmed by ddPCR for DNA on target integration, going from 0.2 to 1 copy per cell with NHEJi. Although being more modest, similar results were observed on murine HSPCs. Noteworthy, we reported that NHEJi could also increase the efficiency of CRISPR-mediated genomic deletion, as indicated by a ddPCR measuring deletion

efficiency between the two cutting sites of our single gRNA. In addition, targeted long-read sequencing is currently on-going to obtain a more detailed analysis of targeted integration and deletion events. Finally, we are testing NHEJi outcome on other genomic loci and its toxicity, as well as its effect in combination with other drugs inhibiting alternative DNA repair pathways. As HDR requires cycling cells, we will evaluate editing outcomes specifically in primitive hematopoietic stem cells, which are predominantly quiescent. Overall, these data highlight the potential of NHEJ inhibition as a promising strategy for tuning genome editing and enhancing DNA targeted integration.

P655

Lipid nanoparticles efficiently deliver the base editor ABE8e for *COL7A1* correction in dystrophic epidermolysis bullosa fibroblasts *in vitro*

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Adenine base editors (ABEs) convert A•T base pairs to G•C base pairs without requiring double-stranded DNA breaks or donor DNA templates. They represent a potentially useful approach for gene editing in the inherited blistering disease dystrophic epidermolysis bullosa (DEB), which results from pathogenic variants in *COL7A1*, leading to dysfunctional or absent of type VII collagen (C7) in the skin basement membrane. We have previously shown that ABE8e, a new variant of ABE, displays remarkable editing efficiency (94.6%), and a favourable safety profile, in correcting pathogenic variants and restoring C7 expression when electroporated into primary fibroblasts from a patient with DEB. Our future goal would be to develop therapeutic topical base editing creams for DEB and therefore we now explore the use of a lipid nanoparticle (LNP) system to deliver ABE8e in mRNA format to effectively edit a pathogenic variant in DEB fibroblasts. Here, we test six different LNP formulations, all of which use the phospholipid DOPE, microfluidically mixed with one of the following cationic lipids: DTDTMA (C14), DHDTMA (C16), or DOTMA (C18). These liposomes are then either mixed with ABE8e mRNA and sgRNA alone, or with ABE8e mRNA, sgRNA and a peptide containing an epithelial targeting motif (K_{16}GACYGLPHKFCG) to create the final library of six lipid-peptide nanocomplexes. Using Sanger Sequencing, we show comparably efficient correction of the pathogenic variants (up to 100%) using formulations containing C14 and C16 lipids, both with and without the peptide. Western blot analysis revealed that the correction of the pathogenic variant in *COL7A1* corresponds to an increase in C7 production and secretion in cell lysates and supernatant, respectively. Additionally, to assess cytotoxicity, a lactate dehydrogenase (LDH) assay was performed which showed that our most efficient formulations induce significantly less cell death compared to lipofectamine. While further safety testing is yet to be carried out, these formulations show great potential for clinical translation and *in vivo* application to treat DEB.

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Allele-specific correction of *GATA2* by ex vivo HDR-based Cas9 genome editing as a therapy for GATA2 deficiency

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GATA2 deficiency is an autosomal dominant disorder caused by variants in the gene encoding the transcription factor GATA2. GATA2 plays an essential role in the self-renewal and differentiation of hematopoietic stem cells (HSCs). As a result, the most common symptom exhibited by patients with GATA2 deficiency is immunodeficiency caused by immune cell cytopenia leading to infections dominated by mycobacteria, herpesviruses, and human papillomavirus. Moreover, patients have a high risk of developing leukemia/hematological malignancies. Here, by nucleofection-based delivery of Cas9/sgRNA RNPs combined with DNA donor delivery by rAAV6 transduction, we demonstrate allele-specific correction of a 7-bp deletion in *GATA2* (c.956_962delGTGGCCT) causing GATA2 deficiency. This gene correction leads to reconstitution of GATA2 expression and function. Using an optimized HDR-based editing protocol with an allele-specific sgRNA, we corrected the variant in more than 99% of the alleles in a homozygous K562 cell line model, leading to the complete reconstitution of the GATA2 protein level, whereas indels were not detected in normal K562 cells. In patient-derived PBMCs heterozygous for *GATA2* c.956_962delGTGGCCT, we showed that this variant was effectively corrected without disruption of the normal allele. DISCOVER-seq was used to nominate potential off-target sites for the most promising allele-specific sgRNA. High-throughput amplicon sequencing of the nominated off-target sites in patient-derived PBMCs revealed one off-target site, located in the long non-coding RNA gene *AATBC*, which was targeted by HiFi Cas9 RNPs with indel frequencies of 5.25%. To estimate the HDR efficacy in $CD34^+$ hematopoietic stem and progenitor cells (HSPCs), we co-delivered Cas9 RNPs with a sgRNA targeting the wildtype *GATA2* sequence and donor DNA harboring silent mutations in the 7-bp sequence to HSPCs from healthy donors, leading to HDR in 72% of the alleles. Using this DNA donor, we found that HDR rescued the colony-forming potential of GATA2 knockout HSPCs observed when the HDR donor was omitted. To investigate the potential selective advantage of GATA2-corrected HSCs *in vivo*, immunodeficient mice were engrafted with mixed populations of HSPCs containing cells engineered to carry either (i) the *GATA2* c.956_962delGTGGCCT variant or (ii) silent mutations in *GATA2*. We found a significant enrichment of non-disruptive mutations after engraftment, thus indicating that HSCs with intact *GATA2* outcompeted HSCs carrying a defective *GATA2* allele.

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Development of a hfCas12Max-based gene editing therapy in a humanized mouse model of Huntington's disease

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Huntington's disease (HD) is a fatal, inherited neurodegenerative disorder caused by the CAG trinucleotide repeat expansion in exon1 of the huntingtin (*HTT*) gene, which leads to the polyglutamine-expanded stretch of mutant Huntingtin (mHTT) protein. The disease involves progressive degeneration of neurons in the striatum and cerebral cortex resulting in chronic movement, psychiatric problems, and cognitive function symptoms that appear during mid-life and slowly progress over the course of two decades until death. Although various approaches have been developed to block mHTT expression and prevent toxic neurodegeneration, there are currently no disease-modifying treatments that can slow disease progression in HD. Knockdown of *HTT* has been reported as an effective way to inhibit the formation of mHTT protein. Here, we present a novel gene-editing approach using guide RNA targeting *HTT* (g*HTT*) through high-fidelity Cas12Max (hfCas12Max), an engineered Cas12i with high editing efficiency and specificity, as a potential treatment strategy for HD. We constructed a single AAV vector packaging hfCas12Max and g*HTT* then bilateral intrastriatal administrated either AAV-hfCas12Max-g*HTT* or PBS to the 3-month-old zQ175 mice, a knock-in (KI) mouse model of HD. We evaluated the indel efficiency, mHTT protein aggregates, and the motor function of the zQ175 KI mice 3 weeks after the injections. In the zQ175 KI mice treated with AAV-hfCas12Max-g*HTT*, the indel ratio of *HTT* reached 67.5% (p=0.062) while the knockdown efficacy of mHTT protein was up to 72% (p=0.0018) in the striatum three weeks after injection when compared to the zQ175 KI mice injected with PBS. Furthermore, zQ175 KI mice treated with AAV-hfCas12Max-g*HTT* showed shorter traverse times on the balance beam, indicating improvements in motor performance at 4 (42.2%, p=0.0792) and 8 (55.51%, p=0.0285) weeks post-injection when compared to the zQ175 KI mice treated with PBS. Our findings using hfCas12Max with higher editing efficiency and specificity than CRISPR-Cas9 demonstrate proof-of-principle of a novel CRISPR-Cas12i system as a therapeutic approach to treat HD, a strategy with implications for the treatment of other neurodegenerative disorders.

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Assessment of safety and feasibility of a Cas13-based RNA editing therapy for *MECP2* duplication syndrome in non-human primates

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Methyl-CpG binding protein 2 gene (*MECP2*) duplication syndrome (MDS) is a rare and fatal childhood neurodevelopmental disorder affecting predominantly males. It is caused by the duplication of the chromosomal region containing the *MECP2* gene, which encodes MeCP2 for proper brain development and maintenance of brain function, resulting in delayed motor

development and severe intellectual disability, ataxia, seizures, language absence or impairment, recurrent respiratory infections and shortened lifespan, with death often occurring before the age of 25 years. Currently, there is no treatments available for this devastating disease. We previously developed a single AAV vector delivering high-fidelity Cas13Y (hfCas13Y) and gRNAs targeting *MECP2* (g*MECP2*). Our experiments using AAV-hfCas13Y-g*MECP2* in humanized MDS mouse model demonstrated the reduction of MeCP2 proteins and reversal of its disease features. Here, we investigated the distribution of hfCas13Y, knockdown of *MECP2* in the brain, and safety in the wild-type (WT) non-human primates. We intracerebroventricularly (ICV) injected low or high doses of AAV-hfCas13Y-g*MECP2* in WT male monkeys. The hfCas13Y was widely distributed in the whole brain of the monkeys with a single injection of either low or high doses of AAV-hfCas13Y-g*MECP2*. In the low-dose group, the *MECP2* knockdown efficiency was more than 20% in 40 percent of the brain regions with maximum knockdown efficiency reaching 70% for the whole brain. In the high-dose group, the mean *MECP2* knockdown efficiency was 52.19 ± 0.0307% with the maximum knockdown efficiency being 85.62% for the whole brain compared to the control group. Furthermore, we found a significant decrease of GDF11, a biomarker of MDS treatment, by ELISA. The blood and pathological results showed good tolerance of AAV-hfCas13Y-g*MECP2* in WT monkeys 8 weeks after ICV injection. Our findings provide a strong foundation and promising therapeutic RNA-editing approach for the clinical use of a single AAV vector delivering hfCas13Y-g*MECP2* in patients with MDS.

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G1 phase synchronization prevents ON-target megabase-scale rearrangements induced by CRISPR-Cas9

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The CRISPR-Cas9 system has revolutionized our ability to precisely modify the genome and has led to gene editing in clinical applications. Comprehensive analysis of gene editing products at the targeted cut-site has revealed a complex spectrum of outcomes. A major concern is the potential genotoxicity of DNA double-strand breaks (DSB), which arise from incorrect or ineffective DNA repair and DNA damage response. ON-target genotoxicity is underestimated with standard PCR-based methods and necessitates appropriate and more sensitive detection methods. Here, we present two complementary Fluorescence-Assisted Megabase-scale Rearrangements Detection (FAMReD) systems that enable the detection, quantification, and cell sorting of edited cells with megabase-scale loss of heterozygosity (LOH). These tools offer highly sensitive readouts to decipher the short-term (murine FAMReD) and long-term (human FAMReD) risk and to find solutions to limit it. They reveal rare complex chromosomal rearrangements caused by Cas9-nuclease and show that LOH frequency depends on cell division rate during editing and p53 status. Cell cycle arrest during editing suppresses the occurrence of LOH without compromising editing. These data were confirmed in human stem/progenitor cells, suggesting that clinical trials should consider p53 status and cell proliferation rate during editing to limit this risk by designing safer protocols. In particular, cell cycle blockade by palbociclib could offer opportunities to make nuclease-based gene therapy protocols safer.

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In vivo pharmacology studies supporting adoptive T regulatory cell therapy for acute respiratory distress syndrome and type 1 diabetes

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T regulatory (Treg) cell therapies have the potential to reestablish immune tolerance in autoimmune diseases. Adoptive transfer of sorted cultured Tregs, although safe, exhibited limited efficacy in previous clinical trials, likely reflecting a lack of tissue homing specificity, limited IL-2 signaling support, and in vivo stability of Tregs. Here, in vivo pharmacology studies utilizing murine engineered Treg cells (mEngTregs) support preclinical development for their use in both acute and chronic indications. Acute indications, such as acute respiratory distress syndrome (ARDS), would require an allogeneic off-the-shelf therapy for immediate dosing, whereas chronic diseases, such as T1D, can use an autologous cell therapy to improve long-term persistence.

mEngTregs were generated for both allogeneic and autologous studies by CRISPR/Cas9-mediated knock-in of a strong promoter into the Foxp3 locus to drive expression of Foxp3 in mouse CD4+ T cells.

For ARDS, we leveraged a Lipopolysaccharide (LPS)-induced model of acute lung injury. Lung injury was induced via intratracheal LPS instillation in C57BL/6 recipient mice and mEngTregs were adoptively transferred one day later via intravenous tail-vein injection. mEngTregs from both allogeneic (CB6F1; C57BL/6 x BALB/c F1, haplotype-matched) and autologous (C57BL/6) donors resulted in significant recovery in body weight, blood oxygen content and lung weights. Additionally, fewer neutrophil infiltrates were present in lung and bronchoalveolar lavage fluid (BALF), lower fibrosis and lesion scores were observed by histopathology, and a trend towards lower inflammatory cytokines was measured by cytokine bead array. Allogeneic mEngTregs were detectable specifically at sites of inflammation (lung and BALF) at days 4-6 and remained in the BALF at day 11, suggesting persistence throughout disease resolution. These data supports development of an off-the-shelf engineered Treg cellular therapy for ARDS.

For T1D, we leveraged adoptive transfer mouse models by dosing either islet-specific monoclonal BDC2.5+ T effector cells or polyclonal diabetogenic splenocytes isolated from hyperglycemic non-obese diabetic (NOD) mice to drive T1D in a T cell dose- and T-cell receptor dependent manner in immunodeficient NOD-scid-IL2rγNULL (NSG) mice. mEngTreg were generated from NOD transgenic mice expressing the BDC2.5 islet specific TCR by genome editing as described above. We demonstrate an effective therapeutic intervention with mEngTreg administered earlier (day 7) or at the time of pancreatic islet inflammation (day 15), preventing progression to diabetes in 100% or 89% of the mice respectively, while all control group developed hyperglycemia by day 42 post-splenocyte transfer. At day 70, treated mice retained glucose control and mEngTregs were present in the pancreas where they decreased the severity of insulitis and preserved the beta cell mass. These data highlight both the direct and bystander suppressive capacity of antigen-specific mEngTreg to prevent or treat T1D.

Thus, we showed in animal models for acute and chronic indications, the benefit of allogeneic and autologous engineered Tregs for treatment of autoimmune and inflammatory diseases.

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Unlocking the potential of designer-recombinases: Improving activity and target site specificity through recombinase engineering

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The precise and seamless mechanism of DNA inversion, integration, or excision makes Cre-type site-specific recombinases (SSRs) appealing for therapeutic applications. Through directed molecular evolution the specificity of Cre can be changed to defined genomic target sequences, enabling the use of SSRs in the context of genetic diseases. However, the step-wise evolution process to alter target site specificity can result in reduced activity or specificity in the evolved variants. To address these limitations and improve the properties of designer-recombinases, we have explored possibilities to fuse designer-recombinases with other protein domains. By applying a nanopore sequencing approach, we developed a fusion architecture that effectively enhanced the activity and target site specificity of designer-recombinases. We demonstrate the applicability of this approach by successfully translating it to different designer-recombinases. Furthermore, when applied to a recombinase previously evolved in our group for correcting the large 140 kb genomic inversion causing hemophilia A, the developed technology successfully improved its properties, positioning it as a preferred candidate for future therapeutic applications. Overall, our study highlights the possibilities to improve the applied properties of designer-recombinases by extending their coding sequences. This approach holds great promise for advancing genetic engineering and exhibits considerable potential for therapeutic applications.

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Assessing safety and genome integrity after CRISPR-Cas9 editing of human hematopoietic stem and progenitor cells for the treatment of *RAG1* deficiency

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Genome editing based on CRISPR-Cas9 is a promising tool that has the potential to cure human genetic diseases. Although the recent advances in the development and applications of CRISPR-based genome editing, evaluation of safety and genome integrity is still required to unfold new methods for the identification of unintended genetic alterations that can potentially occur after editing procedures. We have recently developed a CRISPR-Cas9 long-range gene editing

approach in human hematopoietic stem and progenitor cells (HSPCs) for the treatment of human *recombination activating gene 1* (*RAG1*) defects, a cause of inborn errors of immunity. An effective knock-out/knock-in strategy was established with the aim to disrupt the mutated h*RAG1* gene by non-homologous end joining (NHEJ) and simultaneously insert a codon optimized h*RAG1* corrective cassette by homologous-directed repair (HDR) process. To evaluate the off-target profile of our Hi-Fi Cas9 ribonucleoprotein (RNP) complex, we complemented *in silico* predicted off-target analysis using CRISPOR with the in-cellulo based assay GUIDE-Seq. This analysis, performed in a human cell line, identified two possible intronic off-targets sites. High-throughput sequencing, evaluated in three different human edited HSPCs derived from healthy donors (HD), confirmed the absence of off-target events in relevant genomic loci and a safe profile of the *RAG1* RNP. To investigate the presence of large deletions, we optimized a droplet digital PCR (ddPCR)-based protocol to measure copy non-neutral loss of *RAG1* adjacent genes, *RAG2* and *TRAF6*, in HSPC-derived colony forming units (CFUs). *RAG2* and *TRAF6* are located at a distance of 13 kb towards the centromere and 56 kb towards the telomere respectively. Few deletions were observed for *RAG2*, while more deletions were found for the *TRAF6* gene in RNP-only edited HD-derived HSPCs, potentially indicating chromosomal arm loss. Of note, we observed that delivering our corrective *RAG1* donor cassette, either as adeno-associated virus or integrase-deficient lentivirus, does not increase the frequency of deletion events. In our setting, on-target deletions appear to be significantly more frequent than off-target nuclease events. Comprehensive evaluation of on-target rearrangements, their persistence, and adoption of mitigation strategies are required before clinical translation.

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Investigation of DSB repair mechanisms and AAV Integration into CRISPR-mediated gene disruption: implications for Cas9 variant selection

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CRISPR-Cas9 is a potent tool for targeted gene disruption. In this study, we compared the use of single or paired Cas9 nucleases and paired Cas9 nickases, combined with the same guides RNA (gRNAs), as therapeutic strategies for *in vivo* gene disruption, in the context of a rare metabolic disease. For this purpose, we used AAV8 vectors to deliver the editing tools into mouse livers, and limited Cas9 expression to hepatocytes using a liver-specific promoter. First, we demonstrated

that paired Cas9 nickases, delivered through two independent AAV vectors, exhibited comparable efficacy to individual or paired Cas9 nucleases effectively reducing the target enzyme level. Conversely and as expected, single nicks failed to disrupt the target gene. Interestingly, we observed that paired nick-induced double-strand breaks (DSBs) are likely repaired through the microhomology-mediated end joining (MMEJ) pathway, resulting in heterogenous modifications of variable sizes between the two nicks, with each event occurring at a very low frequency. As previously reported, DSBs induced by single Cas9 nucleases were primarily repaired through the non-homologous end joining (NHEJ) pathway, generating small insertions and deletions. In the case of paired Cas9 nucleases, a precise deletion of the sequence between the two cuts was observed in most cases. Furthermore, we investigated the frequency of AAV integration at the on-target site. During our analysis, we discovered that existing bioinformatic tools for amplicon sequencing analysis consistently underestimated the frequency of AAV integration, as sequencing reads containing vector integrations were frequently discarded or misclassified. To address this issue, we developed a pre-processing pipeline specifically designed to capture and quantify all reads containing AAV insertions. Interestingly, the use of paired Cas9 nickases showed a significant reduction in AAV integration at the target site compared to individual or paired Cas9 nucleases. These disparities may be attributed to the observed differences in the mechanisms involved in DSB repair following nuclease or nickase cleavage. Finally, to facilitate the clinical translation of the paired Cas9 nickases approach, we developed an all-in-one AAV vector encompassing all the necessary elements for nickase-mediated targeted cleavage. Remarkably, even with a reduced injected dose of this system, we maintained the editing efficiency, resulting in a significant therapeutic effect while reducing AAV integration in a dose-dependent manner. Lastly, we assessed the specificity of the selected gRNAs through CIRCLE-seq, CAST-Seq, and long-read nanopore sequencing. Our comprehensive analysis revealed no off-target activity or chromosomal translocations. In conclusion, our study demonstrates that paired Cas9 nickases hold great promise for *in vivo* gene disruption with therapeutic purposes, reducing the frequency of on-target AAV integration and the risk of off-target effects.

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Towards *in vivo* liver directed genome editing for the treatment of the inherited metabolic disorders PFIC-2 and MSUD

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Progressive familiar intrahepatic cholestasis type 2 (PFIC-2) and Maple syrup urine disease (MSUD) are autosomal recessive metabolic disorders. PFIC-2 is caused by mutations in ABCB11 gene encoding the Bile Salt Export Pump. This results in impaired secretion of bile leading to jaundice, pruritus, fibrosis and ultimately to liver failure. PFIC-2 treatment consists in alleviation of pruritus, external or internal biliary diversion or ileal transporter inhibitors. MSUD is caused by impaired activity of the Branched-chain Keto-Acid Dehydrogenase enzymatic complex resulting from mutations in one of the four genes encoding for its subunits (BCKDHA, BCKDHB, DBT, DLD), which leads to accumulation of branched-chain amino acids and their by-products in plasma. MSUD patients are characterized by maple syrup odour in the urine, feeding problems, mental and physical retardation. They are managed with a protein restricted diet to avoid

metabolic decompensation. For both diseases, liver transplantation has been shown to be beneficial. For this reason, liver directed *in vivo* genome editing may represent an alternative to liver transplantation, potentially allowing for stable gene correction in PFIC-2 and MSUD paediatric patients. Here, we aim to evaluate the efficiency of a CRISPR-Cas9 platform for integrating ABCB11 and DBT cDNA under the control of their endogenous promoters, by delivering the donor DNA *in vivo* with viral vectors. We designed guide RNAs (gRNA) targeting the first intron of *ABCB11* and *DBT* to address most of the disease-causing mutations and maintain as much as possible physiological gene regulation. We screened 12 gRNAs for each gene based on their cutting efficiency in hepatocyte cell lines and selected the best performing ones. We then tested different integration strategies, exploiting mCherry as reporter gene, based on homology directed repair (HDR), micro-homology mediated end-joining and homology-independent targeted integration (HITI). *In vitro*, we observed the highest integration rate with HDR-based donor DNAs. On the other hand, when we systemically administered adeno-associated viral vectors (AAV) delivering the different donor DNAs to Cas9 transgenic mice, we observed higher percentage of integration exploiting the HITI-based strategy (36.9%) compared to the HDR-based one (22.6%). In the context of PFIC-2, we performed additional experiments aimed to evaluate the impact of liver fibrosis on viral vector transduction. This is particularly relevant for a disease like PFIC-2, in which the early onset of liver fibrosis could impact on the delivery of the genome editing components and thus on the efficacy of the treatment. To this aim, we intravenously administered lentiviral vector (LV) or AAV encoding for a reporter gene to CCl_4 treated mice, displaying severe pericentral fibrosis. In this model we observed reduced LV transduction while AAV transduction was not affected. In mice treated with DDC diet, displaying severe periportal fibrosis, we observed reduced transduction efficiency for both LV and AAV. Moreover, we intravenously administered LV to 6-month-old PFIC-2 mice, displaying mild periportal fibrosis and showed a slight reduction of transduction. Overall, these data provide the basis to further develop *in vivo* genome editing approaches and to assess the best therapeutic window for the treatment of PFIC-2 and MSUD patients.

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COL7A1 repair via prime editing in dystrophic epidermolysis bullosa

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Epidermolysis bullosa (EB) is an inherited monogenetic blistering skin disease caused by mutations in genes encoding structural proteins within the basement membrane zone (BMZ) of the skin, separating the epidermis from the underlying dermis. Malfunction or absence of one of these proteins result in a severe phenotype, including massive blistering and skin loss, high risk of infections and the development of skin cancer. In this study we used prime editing (PE) to target a splice site mutation (425A>G) within the *COL7A1* gene, causing recessive dystrophic EB (RDEB), with the aim to restore type VII collagen (C7) function in RDEB keratinocytes.

Therefore, RDEB keratinocytes were transfected with plasmids carrying the Cas9 nickase (Cas9n)-Reverse transcriptase (RT) fusion protein, the prime editing gRNA (pegRNA) and an

additional sgRNA to nick the non-edited strand in order to increase the editing efficiency (PE3b). Blasticidin selection was performed to select for plasmids uptake. The PE-treated cell population was then analyzed via immunofluorescence staining (IF), flow cytometric analysis, Western blot (WB) analysis and next-generation sequencing (NGS). In addition, 3D skin equivalents were generated from gene-edited patient keratinocytes and untreated RDEB fibroblasts. IF stainings and flow cytometry revealed the presence of ~22.78% of patient cells re-expressing C7 upon PE treatment. This is emphasized by WB images from cell lysates and supernatants, both revealing the presence of C7 in treated cells. Furthermore, NGS analysis indicated 12.41% of corrected *COL7A1* alleles. More importantly, no insertions or deletions (indels) were detected at the on- and *in silico* predicted off-target sites. The 3D skin equivalents showed the presence of a continuous C7 layer within the basement membrane zone between dermis and epidermis, indicating the accurate reversion of the disease phenotype.

In summary, PE is a highly accurate tool for traceless correction of monogenetic diseases. Our data shows that PE reactions are accompanied by low amounts of bystander products, a fact that sets it apart from other CRISPR/Cas9-based editing techniques. Although still work-in-progress, our initial experiments detailed here already highlight the suitability of PE as a gene editing tool for EB as well as for other genodermatoses.

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A prime editing toolkit delivered using lipid nanoparticles for precise gene editing of human inherited cardiomyopathies

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Dilated cardiomyopathy (DCM) is an inherited cardiac heart condition that progressively worsens contractility of the myocardium, leading to left ventricular wall dilation and heart failure. Since most cases are characterized by dominant inheritance, precise gene editing to correct the underlying mutations would be a most direct strategy to find a cure. However, CRISPR-Cas9 knock-in through canonical homology directed repair has very limited applicability, as homologous recombination is ineffective in post-mitotic cardiomyocytes. A possible alternative is prime editing, which does not require double stranded DNA breaks and is based on the substitution of a short stretch of DNA across a mutation through reverse transcription of template RNA. However, prime editing efficiency still needs to be improved for in vivo applications.

Using a U2OS stable cell line expressing a mutated form of the EGFP (Y66S) to detect prime edited cells, we performed a high throughput screening using a whole genome library consisting of 2602 human miRNAs mimics. The screening identified two miRNA families that significantly increase prime editing efficiency. These miRNAs are also effective in primary neonatal mouse ventricular myocytes while the mechanism by which these RNA molecules can increase prime editing efficiency is currently under investigation. Our intention is to use the most effective of these miRNAs as adjuvants that transiently increase the efficiency of cardiac prime editing in vivo.

To deliver the prime editing components and the adjuvant miRNAs to the heart, we developed both viral and non-viral delivery methods. The viral methods are based on AAV vectors that transfer both the pegRNA (containing the CRISPR guide and the RNA template for correction) coupled with the engineered Moloney Leukemia virus reverse transcriptase and Cas9 nickase

using two distinct AAVs. The non-viral methods are based on lipid nanoparticles (LNPs) formulated according to the SNALP technology, which we optimized for cardiac specific delivery. The prime editing toolkit LNP packages, within in the same particle, the prime editor mRNA, obtained by in vitro transcription, an engineered pegRNA (e-pegRNA) and one of most efficient miRNA that boost efficiency of prime editing. We sought to use these methods for the correction of a mutation in exon 9 (R636Q) of the RBM20 gene, which is a frequent cause of dilated cardiomyopathy and for which a humanized knock in mouse model is available.

Our results indicate that prime editing can be enhanced by small non coding RNAs in neonatal cardiomyocytes in vitro and that we established both viral and non-viral delivery methods as promising tools to utilise this technique in vivo.

P667

Comprehensive analysis of TALE-BE editing determinants

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TALE base editors (TALE-BE) are fusions of a transcription activator-like effector domain (TALE), split-DddA deaminase halves, and an uracil glycosylase inhibitor (UGI). These recent additions to the genome editing toolbox have the ability to directly edit double strand DNA, converting a cytosine (C) to a thymine (T) through the formation of an uracil (U) intermediate. Here we present a comprehensive investigation of key fundamental characteristics of C-to-T TALE-BE functions within the nuclear genome.

We recently developed a medium to high throughput *in cellulo* TALE-BE screening strategy that excludes confounding factors such as epigenetic and microenvironmental differences. Using this strategy, we demonstrated that the nature of the domain linking the TALE binding domain and the split deaminase allowed to tune C-to-T conversions within the editing window. The robustness and versatility of this approach allowed to gain in depth insight of the editing rules *in cellulo* and further highlighted that the composition surrounding the TC to be edited could strongly impact editing efficiencies. Therefore, educated choice of the TALE-BE architecture and positioning on DNA (spacer length) could either prevent target sequence limitations (increasing targetable sequence space) or, on the contrary, could be used to decrease, if not eliminate, bystander editing within the editing window, allowing for more precise genome editing outcomes.

Next, we used *in silico*-based (bioinformatic) tools and wet laboratory-based approaches to assess potential nuclear off-site editing. This strategy based on TALEN® (TALE nuclease) led us to identify potential off-targets that were further assessed using targeted multiplexed PCR. We additionally used a custom DNA target enrichment process to assess hundreds of sites simultaneously, overall allowing to demonstrate, in our experimental conditions, the absence of editing on sites that were previously described to be pan-TALE-BE off-site hot-spots.

We believe that the knowledge presented will help ensuring that genome editing-based therapies are efficiently and safely designed and minimize the risk of genotoxic events while overall expanding the potentials for nuclear and mitochondrial therapeutic cell engineering.

P668

Engineered SpCas9-azide enables functional codelivery of click-conjugated siRNA

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Gene correction using CRISPR systems is currently a hard goal to achieve. Great strides were made in CRISPR gene correction by engineering the SpCas9 protein to exploit novel routes of gene correction, such as fusion proteins like base-editors and prime-editors. The design space for SpCas9 protein fusion is however limited to the C-terminus due to the presence of active sites near the N-terminus. This limitation prompted the current study, in which we aimed to develop SpCas9 containing an azide handle for click-chemistry on the protein surface, with the main aim of retaining protein activity after bioconjugation to macromolecules.

First we identified regions on the protein surface based on earlier literature, in which SpCas9 can be modified without loss of activity. We chose to substitute aromatic amino acids with high solvent accessibility in the Cas9 crystal structure in these regions with p-azido-phenylalanine (pAzF) as click-chemistry handle, which is introduced by STOP codon reprogramming in *E.Coli*. Eleven potential substitutions were designed *in* silico, four of which were generated and characterized *in* vitro. One variant in particular, 539pAzF-Cas9, retained bioactivity after conjugation of fluorescent dye or polyethelyne glycol to the protein. This variant was used in further studies.

Any molecule containing an alkyne group can be clicked to 539pAzF-Cas9, leading us to choose siRNA as a potentially relevant and tricky model molecule. siRNA is large (~14 kDa), poly-anionic and potentially unstable. We functionalized siRNA first by attaching a linker molecule which enables release of the native siRNA in reducing environments such as the cytosol. This linker was further functionalized with a ring-strained alkyne (TMTHSI), which enables copper-free click conjugation of the siRNA to the SpCas9 surface.

In a proof of concept study we demonstrate that Cas9-siRNA complexes are generated with a 50% conjugation efficiency based on gel electrophoresis and densitometry analysis. The siRNA is released from the protein in presence of 5 mM glutathione which mimics intracellular conditions. Furthermore we show that in model cell lines for Cas9 and luciferase siRNA activity (HEK293t expressing eGFP and dual luciferase respectively), both components are active after delivery using commercial lipofection kits.

In conclusion 539pAzF-Cas9 enables site-directed click-conjugation of a large molecular payload (siRNA) which does not detract from the SpCas9 function in cells and can deliver the siRNA to the cytosol successfully. This can be used in future experiments to enhance CRISPR therapy by codelivery of cargoes through surface conjugation, rather than limiting this to protein fusions.

P669

Optimizing gene therapy to cure DADA2 deficiency

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Adenosine deaminase 2 deficiency (DADA2) is an ultrarare autoinflammatory disorder, caused by a mutation in the *ADA2* gene. Patients suffer from autoinflammation with dysregulated immune function, bone marrow failure, hematologic abnormalities and in severe cases neurological deficits and injury to the kidney. Anti-TNF treatment suppresses autoinflammation and vasculitis in these patients but does not resolve all symptoms. Another treatment is hematopoietic stem cell transplantation, but this has great risk of causing Graft versus Host Disease. Gene augmentation therapy is still in development, but would not tightly regulate *ADA2* expression, possibly causing other symptoms. Now, using our fluoPEER system, we have successfully screened prime editing constructs and reach high levels of correction of the most common *ADA2* mutation. Additionally, we are expanding hematopoietic stem cells and have proven multiple successful delivery methods of our prime editing constructs. These include generation of modified RNA, engineered virus-like particles, and lipid nanoparticles, which all have proven to correctly edit multiple mutations in our lab. Prime editing-based correction of DADA2 hematopoietic stem cells would avail autologous stem cell transplantation or *in vivo* gene correction, allowing full patient recovery with minimal side effects.

P670

Gene Expression Modulation Systems (GEMs): A CRISPR-Based Epigenome Editing Platform for In vivo therapeutics

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Epigenome editing holds immense potential for programmable modulation of gene expression, enabling therapeutic applications. However there remain significant barriers to realizing this potential including in delivering bulky and highly active epigenome editing platforms *in vivo*. In this study, we present the development of a highly optimized CRISPR-based epigenome editing platform termed Gene Expression Modulation systems (GEMs). Through extensive engineering efforts, we have made notable improvements in Cas proteins, guide RNA scaffolds, and added the discovery of novel compact modulator proteins. Our platform integrates these advancements to achieve precise and efficient control over gene expression. Firstly, we have systematically identified and characterized thousands of compact modulators capable of transient and persistent gene regulation. These compact modulator proteins serve as effective tools for manipulating gene expression, allowing fine-tuning of specific target genes in a programmable manner. Additionally, we have engineered a CRISPR-Cas system to enhance the functionality of the ribonucleoprotein (RNP) complex for more efficient epigenetic editing. Through these modifications, we have achieved a more compact RNP complex that exhibits improved editing activity, thus enhancing the precision and efficacy of our epigenome editing system. Overall, our GEMs provide a versatile and powerful tool for a broad range of epigenome modulation and gene therapy applications. The

optimized CRISPR-based platform, combined with the extensive repertoire of compact modulator proteins, enables precise control over gene expression, opening new avenues for therapeutic interventions and potential cures for genetic disorders *in vivo*.

P671

Highly efficient Base Editing correction in *COL7A1* gene in primary RDEB keratinocytes.

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Recessive Dystrophic Epidermolysis Bullosa (RDEB) is a severe skin disease caused by mutations in *COL7A1* gene, leading to absent or dysfunctional type VII collagen (C7) and, consequently, to chronic blistering, progressive fibrosis, and high risk of developing cancer. We have identified in our cohort two patients with *COL7A1* mutations located in exons 72 and 94, due to a C>T substitutions, leading to premature termination codons.

Here we have tested whether Adenine Base Editors (ABE) could correct these mutations and restore C7 in primary RDEB keratinocytes lacking this protein. Delivery of base editing tools was achieved by nucleofection of ABE8e mRNA and guide.

For the mutation in exon 72, we obtained editing efficiencies of 90 ± 8% in primary RDEB keratinocytes, removing the premature stop codon. As expected, predicted bystander modifications were generated (with editing rates of 93 ± 4%), although we have confirmed *in silico* and experimentally that the wild type amino acid sequence remains since the modifications involved just silent mutations. Hence, the C7 expression has been successfully restored in these primary RDEB keratinocytes, as evidenced by immunofluorescence and western blot assays.

On the other hand, in RDEB keratinocytes from the patients with the mutation (heterozygous) in exon 94, the editing rates were 80 ± 13%. Moreover, as in the previous case, the predicted bystander modifications (with editing rates of 65 ± 22%) had no impact in the amino acid sequence. Again, immunofluorescence and western blot assays confirmed that the expression of C7 is recovered to levels high enough to correct the disease.

Ongoing experiments aim to demonstrate the performance of skin-equivalents prepared with these efficiently modified patient keratinocytes in *in vivo* mouse models.

P672

Miniature base editors using engineered diverse IscB proteins

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As a miniature RNA-guided endonuclease, IscB is likely the ancestor of Cas9 and shares similar functions. Given the large size of Cas9, it has proved challenging to deliver the protein into target cells, a feature reflecting the packaging limit associated with the delivery of viral vectors such as adeno-associated virus (AAV). Therefore, delivering genes encoding Cas9, a single guide RNA (sgRNA), and a donor DNA together with associated promoters into a single AAV vector remains a major obstacle for genome editing therapy. On the contrary, IscB is less than half size of Cas9 and thus more suitable for *in vivo* delivery by a single AAV vector. However, the poor editing efficiency of IscB in eukaryotic cells limits its *in vivo* applications and has not been addressed. The IscB protein has been shown to work as a programmable RNA-guided endonuclease similar to Cas9 nuclease, while also requiring a target-adjacent motif (TAM) in the target dsDNA. To improve the editing efficiency, we engineered the OgeuIscB protein and its corresponding ωRNA, a non-coding RNA, with multiple mutagenesis screenings to generate the highly efficient OgeuIscB system with 5'-NWRGNA-3' (TAM) recognition. A fluorescent reporter system was used to assess the activity of IscB system by fluorescence intensity. Firstly, we engineered the ωRNA based on its secondary structure and obtained the optimal ωRNA version. Then, we mutated each nonpositively charged amino acid in IscB into positively charged arginine and combined with different mutations to obtain IscB variants with the highest editing efficiency. We further obtained IscB nickases by mutating the catalytic amino acid of the RuvC domain to alanine. By fusing cytosine deaminase, adenosine deaminase, or MPG glycosylase with enhanced IscB nickases, we generated miniature IscB-derived base editors (miBEs), exhibiting robust editing efficiency (up to 92%) to induce DNA base conversions. Furthermore, we also conducted a systematic evaluation of the specificity of IscB-derived base editors and found that their specificity was comparable to SpG Cas9. Finally, we identified several new variants of IscB with different TAM sequences, broadening the target range of miBEs. Overall, our work establishes miBEs as versatile tools for efficient genome editing based clinical applications.

P673

HG-PRECISE platform improves the performance of RNA-guided nucleases

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HuidaGene – **P**latform for **R**ational **E**ngineering of **C**RISPR-Cas **I**dentification by **S**ynergic **E**xpertise (HG-PRECISE) platform is a directed evolution system that works in mammalian cells based on a triple fluorescence reporting vector. Proteins evolved from non-mammalian cells may not work directly in mammalian cells due to several factors such as misfolding and incorrect cell localization. Therefore, HG-PRECISE has great advantages in

engineering genome editing proteins, such as Cas proteins, used in mammalian cells. In this project, we used HG-PRECISE platform to engineer several Cas12, Cas13, and IscB proteins, including RxCas13d, Cas13X.1, Cas13Y.1, xCas12i, OsCas12f1, RhCas12f1, and OgeuIscB. Through this platform, we obtained various nucleases with improved specificity, robust editing efficiency, and broader targeting ability. Through structure-guided efforts using the HG-PRECISE platform, we generated hfCas13d, hfCas13X.1, and hfCas13Y.1 with reduced bystander activity by hundreds of folds. After multiple mutagenesis screenings over 500 mutants on xCas12i, we identified several mutation sites which have the function of expanding PAM recognition and improving specificity. By combining these mutations, we developed hfCas12Max with high specificity, high activity, and recognition of 5 '- NTN PAM. OsCas12f1 and RhCas12f1 are the smallest Cas12 proteins with only about 400 amino acids, and recognize PAM of 5 '- TTN and 5' - CCD, respectively. However, the editing efficiency of the wild-type Cas12f is relatively low, which limits its applications. Through protein and sgRNA engineering by HG-PRECISE, we generated enhanced enOsCas12f1 and enRhCas12f1 variants with up to 99% editing efficiency. IscB is a miniature RNA-guided endonuclease that is presumed to be the ancestor of Cas9 and shares similar functions. The poor editing efficiency of IscB, however, limits its *in vivo* applications. Using HG-PRECISE, we developed an enIscB system composed of engineered OgeuIsCB and its corresponding ωRNA, which is highly efficient in mammalian systems. Therefore, the HG-PRECISE platform is highly versatile and powerful for improving the performance of RNA-guided nucleases. Continued development of the HG-PRECISE platform will likely lead to smaller, more robust, and more efficeint versions of CRISPR base editing, prime editing, and epigenetic editing technologies.

P674

Exploring the potential of a novel Cas13-based RNA-targeting therapy for age-related macular degeneration

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Age-related macular degeneration (AMD) is a progressive disease leading to severe impairment of central vision and is a widespread cause of visual impairment among adults over the age of 50. AMD can be categorized into two main groups: dry or non-neovascular AMD, and wet or neovascular AMD. Neovascular AMD (nAMD) presents with the excess blood vessel growth and leakage, accounting for 90% of AMD-related cases of blindness. nAMD is primarily driven by the perturbation of vascular endothelial growth factor (VEGF), an angiogenic growth factor, which plays a crucial role in pathological angiogenesis. Although anti-VEGF therapies for nAMD are both effective and safe, their long-term efficacy tends to wane as repeated intravitreal injections compromise patient adherence to treatment regimens, potentially resulting in vision loss in the 7th or 8th year of treatment. Additionally, frequent intravitreal (IVT) injections increase the likelihood of complications, such as submacular hemorrhage, intraocular hypertension, glaucomatous optic neuropathy, endophthalmitis, and retinal detachment. Recently published in *JAMA Ophthalmology*, a large-scale 5-year study of more than 1.7 million patients reported an association between IVT injections of anti-VEGF and systemic adverse events. Despite the wide-scale use of anti-VEGF therapies, long-term clinical data suggests that VEGF-targeted therapy can be limited by the return of visual decline after repeated invasive administrations over time, indicating a loss of efficacy following an initial response. Furthermore, up to 30% of patients with nAMD do not respond at all to the any standard anti-VEGF therapies as published in *New*

England Journal of Medicine. Our team has developed a Cas13-based RNA targeting therapy, HG202, by packaging a single AAV vector with high-fidelity CRISPR/Cas13 RNA targeting technology to effectively reduce the expression of VEGFA within the local retina.
39% *Vegfa* mRNA expression was reduced after a single subretinal injection of HG202 in mouse eyes ($p<0.0001$). Additionally, the area of choroidal neovascularization induced by laser in mouse eyes significantly decreased by 73% ($p<0.0001$) when compared to the vehicle control, whereas aflibercept only reduced it by 57% ($p<0.0001$). Within the effective dose range, HG202 does not cause retinal structural and functional impairment. This innovative approach allows for precise and efficient gene knockdown with the potential for the patients to reduce frequent IVT injections and minimize the risk of complications, providing a promising therapeutic strategy to ameliorate the social and economic burdens as well as to improve safety profile associated with chronic anti-VEGF therapy by providing reliable and safer option for nAMD. More importantly, HG202 offers a new approach for nAMD patients who are non-responsive to IVT anti-VEGF therapies.

P675

Precise Editing of Primary MicroRNA Reprograms Adipose-Derived Stem Cell Differentiation and Augments Tissue Regeneration

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RNA editing has captured growing interests for potential treatments of genetic diseases and temporary maladies. Current RNA editing methods mostly exploit CRISPR Cas13 or oligonucleotide to guide ADAR (adenosine deaminase acting on RNA) to catalyze adenine (A)-to-inosine (I) base conversion in mRNA transcripts. However, whether these methods can edit microRNA (miRNA) with higher order secondary structure has yet to be explored. Here we developed a miRNA base editor (miRBE) to edit the precursor microRNA (pre-miRNA) hairpin on the primary miRNA (pri-miRNA), thus abolishing miRNA expression in a programmable and precise fashion. We fused different RNA-targeting Cas13 proteins with $ADAR2_{DD}$ via various linker peptides and generated crRNA spacers of different lengths to target at the vicinity of stem base of the pre-miRNA. We uncovered that the Cas13 protein type, linker types and targeting sites on the pri-miRNA remarkably affected the efficiency of A-to-I conversion on various pri-miRNA. The optimized miRBE, consisting of dCas13d and $ADAR2_{DD}$(E488Q) fused with XTEN linker, conferred »10-20% editing efficiencies of various pre-miRNA in HEK293FT, cancer cells and adipose-derived stem cells (ASC), which knocked down the miRNA levels for >50% across different cell types. In particular, miRBE editing of pre-miR-21 in ASC suppressed the miR-21-5p levels for 72%, which repressed the ASC differentiation towards adipogenic lineage while stimulating chondrogenic differentiation with minimal cytotoxicity, off-target effects and perturbation of transcriptome. The pre-miR-21 editing in ASC improved in vitro cartilage formation and augmented calvarial bone regeneration in vivo. The miRBE offers a new opportunity to edit biologically important miRNAs and promote tissue regeneration.

P676

A European effort for therapeutic gene editing in Rett syndrome: from organoids to mouse models

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Rett syndrome is a rare neurodevelopmental disorder and the second most common cause of intellectual disability in women after Down syndrome. Classic form of the disease is caused by MECP2 mutations. Reactivation of Mecp2 in symptomatic KO mice can revert disease phenotypes, suggesting that RTT is not irreversible. Gene editing represents an extremely promising therapeutic approach, since it allows maintaining the endogenous regulatory framework. The approach recently made its way to clinical trial for Leber Congenital Amaurosis (NCT03872479), demonstrating its translational potential. To validate its applicability to the 4 most common MECP2 hotspot variants we established an international consortium that was funded from the EU in 2021. We engineered a CRISPR/Cas9 gene editing toolkit composed of a dual plasmid system specific for each MECP2 mutation. We collected RTT samples and reprogrammed iPSCs from 2 patients for each variant to generate brain organoids, 3D structures which closely mimic human brain development. RTT organoids have been generated for two mutations and characterization is nearing completion. In RTT iPSC-derived neurons and 3D models we are also validating the infection efficiency of the available chimeric AAV7m8 virus that has been chosen for the in vitro experiments. However, these viruses are still far from optimal since a significant portion spreads to peripheral organs reducing the efficiency of the approach and increasing the risk of toxic effects. For this reason, new chimeric serotypes with higher specificity and selectivity will be designed during the project. We validated the correction system efficiency in patient cells confirming the functionality of the tested plasmids with a high efficiency. NGS analysis in fibroblasts obtained from MECP2 patients, demonstrated a high editing efficiency, in particular up to 90% and 72% of HDR for R306C and for R168X variants respectively. Colonies of KI mice harboring 3 variants are also being established to further test the approach in vivo. The phenotyping pipeline has been defined and behavioral characterization is almost complete. Our work will represent a real opportunity for the application of gene therapy for RTT, thus providing the first real possibility for a RTT therapy.

P677

Mutation-specific reporter for optimization and enrichment of prime editing

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Prime editing is a versatile genome-editing technique that shows great promise for the generation and repair of patient mutations. However, some genomic sites are difficult to edit and optimal design of prime-editing tools remains elusive. Here we present a fluorescent prime editing and enrichment reporter (fluoPEER), which can be tailored to any genomic target site. This system rapidly and faithfully ranks the efficiency of prime edit guide RNAs (pegRNAs) combined with any prime editor variant. We apply fluoPEER to instruct correction of pathogenic variants in patient cells and find that plasmid editing enriches for genomic editing up to 3-fold compared to conventional enrichment strategies. DNA repair and cell cycle-related genes are enriched in the transcriptome of edited cells. Stalling cells in the G1/S boundary increases prime editing efficiency up to 30%. Together, our results show that fluoPEER can be employed for rapid and efficient correction of patient cells, selection of gene-edited cells, and elucidation of cellular mechanisms needed for successful prime editing.

P678

Development of a hfCas12Max-mediated gene editing therapy in a humanized mouse model of Amyotrophic lateral sclerosis

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease affecting ~2-5 per 100,000 people worldwide. The hallmark of 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 to 5 years of symptom onset mainly due to the respiratory failure. 97% of ALS patients exhibit cytoplasmic aggregation of mutated TDP-43 protein, an DNA/RNA-binding protein essential for normal functions of the motor neurons, leading to neuron death. *ATXN2* promotes the formation of stress granules involving TDP-43 protein. Knockdown of *ATXN2* may effectively inhibit the formation of TDP-43 stress-granule aggregates. Here, we report the use of gRNA targeting *ATXN2* (g*ATXN2*) through high-fidelity Cas12Max (hfCas12Max), an engineered Cas12i with high activity and specificity, as a potential treatment strategy for ALS. We developed a single AAV vector packaging hfCas12Max and g*ATXN2* capable of accessing neurons in the brain and spinal cord when administered through sequential intravenous (IV) and intracerebroventricular (ICV) injections in neonatal mice. Separate IV and separate ICV injection of AAV-hfCas12Max-g*ATXN2* were also used as controls. The lifespan of humanized *TDP-43* transgenic mice injected with AAV-hfCas12Max-g*ATXN2* or PBS was observed daily for up to postnatal 35 days. Median survival of humanized *TDP-43* mice injected with AAV-hfCas12Max-g*ATXN2* was 34.33 ± 0.7601 (p =0.0006) days by sequential IV and ICV injections when compared to 29.5±0.8660 days by IV injection or 30.75±0.4787 days by ICV injection. Importantly, median survival of *TDP-43* mice injected with PBS was only 24.63±0.5325

days. Therefore, median survival of humanized *TDP-43* mice injected with AAV-hfCas12Max-g*ATXN2* by IV and ICV was the longest compared with the other groups, representing a 39.4% significant extension of survival. Our findings provide a promising and safe DNA-editing approach for therapeutic therapy using AAV- hfCas12Max-g*ATXN2* to treat and potentially prolong lifespan of patients living with ALS.

P679

Profound and durable silencing of PCSK9 in non-human primates by epigenome modulation

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Advancement in gene editing technology has brought hope to combat genetic diseases. However, its application in treating chronic diseases is mainly hindered by the potential safety concern caused by gene sequence changes. Epigenome editing, accomplished by fusing catalytically dead Cas9 (dCas9) with gene-regulatory proteins, offers a compelling alternative to classical cleavage-dependent gene editing. By establishing both DNA methylation and repressive histone modifications at the target sites, epigenome editing achieves sustainable gene silencing. We have developed and optimized an epigenetic modulation system, EPIREG. In this study, we applied lipid nanoparticles (LNPs) to deliver RNA that encoded EPIREG mRNA and sgRNA to the liver tissue or hepatocytes in corresponding species. We observed that a single dose of EPIREG administration to normal mice induced a dose-dependent reduction of both hepatic Pcsk9 mRNA and circulating PCSK9 protein levels, with almost complete knockdown of the target gene at safe and efficacious doses. We further confirmed the specificity of EPIREG by whole-genome bisulfite sequencing and RNA-seq analyses. We also observed that Pcsk9 gene silencing by EPIREG was maintained through hepatocyte proliferation following partial hepatectomy. This was accompanied by increased methylation levels at the Pcsk9 promoter in the liver tissue. In addition, we demonstrated the profound effect and durability of EPIREG in lowering serum PCSK9 and LDL-c levels in a high-fat-diet-induced hypercholesterolemic disease model. Furthermore, this effect was reproduced in cynomolgus monkeys using the relevant species-specific sgRNA selected from primary monkey hepatocytes. Thus, the robust and stable reduction of PCSK9 gene expression and LDL-c levels were induced in non-human primates, without significant adverse effects, following single dose administration of mEPIREG to monkeys. Additionally, in human liver cancer cell line Huh7, the delivery of hEPIREG resulted in a dose-dependent knock-down in PCSK9 gene expression by up to 99.7%, and this inhibitory effect was maintained through dozens of cultivation generations. In summary, our findings strongly suggest that transient epigenome modulation without DNA sequence alteration, can produce robust and long-lasting gene silencing in different species. This offers a potential therapeutic strategy for a wide range of chronic disorders.

P680

Achieving CAR-T cell generation in a single intervention using a multiplex base editing system: harnessing the modularity of the Pin-point™ base editing platform

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The negative consequences of multiplex gene editing using traditional CRISPR-Cas and the advantages offered by base editing are well described in the literature. However, even multi-gene knock-out does not provide the ultimate cell or therapy in a single intervention. Herein we describe the exploitation of the aptamer-based recruitment mechanism of our Pin-point base editing platform to achieve simultaneous multi-gene knockout and CAR insertion to create CAR-T cells in a single reaction. We also show the advantage of this platform in iPSCs, which are known to be sensitive to gene editing.

The Pin-point™ base editing system is a modular technology where the CRIPSR-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 Pin-point system allows for high flexibility in the customization of each individual component to address specific editing needs and enables complex genetic modifications. Additionally, by swapping aptamers and their complementary aptamer binding proteins, different effector molecules could be specifically directed to different genomic loci allowing a suite of independent operations while utilising a single Cas module and avoiding gRNA crosstalk. This opens the possibility of specifically editing each site in a different way. And by removing the aptamer and therefore the deaminase recruitment, the CRISPR-Cas component can be used to allow for gene insertion at the DNA target site. This unique modularity provides unparalleled editing flexibility and significantly increases the complexity of simultaneous edits that are achievable. And by using the same CRISPR-Cas, it minimizes the size of the payload that needs to be delivered.

Here we provide an example of one configuration of the Pin-point technology for the streamlined generation of allogeneic CAR-T cells and hypoimmunogenic iPSCs. By delivering Rat APOBEC1 fused to the MS2 coat protein (MCP), SpCas9 nickase and gene specific gRNAs containing the bacteriophage MS2 RNA aptamer, we achieve high knockout efficiency and editing purity at multiple sites simultaneously. At the same time, an aptamer-less gRNA was used to introduce a targeted transgene knock-in in both human primary T cells and iPSCs. Site-specific knock-in and multiplex gene knockout are achieved within a single intervention and without the requirement for additional sequence-targeting enzymes for the streamlined generation of allogeneic cell models.

The demonstrated ability to perform complex genome editing in multiple cell types safely, efficiently, and precisely opens the door to applying the Pin-point system in a range of advanced cell therapies.

P681

CRISPR-Cas12a gene editing with linear single-stranded DNA repair templates with end modifications enables highly efficient knock-ins of therapeutic transgenes in primary human T cells

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Non-viral gene editing allows the insertion of transgenes in defined locations of the human genome, which can be used to engineer potent cellular therapies. Currently, the most commonly used non-viral template format to induce homology-directed repair (HDR) after nuclease-induced DNA breaks is double-stranded DNA (dsDNA). However, electroporation of high doses of dsDNA is toxic to many primary cell types, including T cells. Consequently, several platforms utilizing single-stranded DNA (ssDNA) templates have emerged to mitigate toxicity and reduce the risk of unintended integrations. Although insertion of large transgenes using linear ssDNA typically yields low knock-in efficiencies, hybrid ssDNA templates harboring short dsDNA-end modifications containing truncated Cas-target sequences (tCTS) have been shown to enhance knock-in rates during editing with the Cas9 nuclease from *Streptococcus pyogenes*. These end modifications are intended to increase ssDNA delivery into the cell nucleus by functioning as binding sites for the Cas protein, which contains nuclear localization signals. To date, hybrid ssDNA with tCTS (ssCTS) have not been adapted for other programmable nucleases, such as Cas12a.

Here, we developed a ssCTS platform to enhance gene editing with the CRISPR-Cas12a Ultra system during reprogramming of T cells with chimeric antigen receptors (CARs) and T-cell receptor fusion constructs (TRuC). To this end, we tested various Cas12a binding motifs with a range of potential mismatches to the guide RNA and different PAM orientation. To exclude locus-specific bias, the screening of tCTS designs was performed for knock-in at three distinct loci of the T-cell receptor-CD3 complex genes, namely *TRAC*, *CD3ζ* and *CD3ε*. After identification of the optimal tCTS sequence, we generated ssDNA from linear dsDNA by enzyme-mediated removal of one DNA strand. Through annealing of tCTS-containing DNA oligos, we generated the ssCTS donor templates. Finally, we compared the efficacy and toxicity of different non-viral DNA templates (dsDNA or ssDNA with or without tCTS) at increasing equimolar concentrations. Independent of the targeted locus or template concentration, ssCTS increased the relative knock-in rates by at least 2-fold (range: 2-fold to 11-fold) in comparison to unmodified ssDNA. At the highest tested template concentration (100nM), ssCTS significantly outperformed dsDNA in respect to knock-in rate and overall yield of reprogrammed cells. At the *CD3ε* locus, we achieved exceptionally high knock-in rates, detecting the 800 base pair transgene in up to 90% of the edited T cells whilst maintaining comparable cell survival to non-edited electroporated T cells. Furthermore, we did not observe indiscriminate cleavage of our ssCTS donors after incubation with crRNA-Cas12a Ultra complexes in vitro.

In conclusion, we demonstrate that tCTS-end modifications in ssDNA donors significantly improves knock-in of therapeutically relevant transgenes when co-electroporated with CRISPR-Cas12a ribonucleoproteins in primary human T cells. Due to lower toxicity of ssCTS templates than dsDNA, this non-viral gene editing platform may be used to optimize reprogramming efficacy to improve manufacturing of CAR T cell therapies and other immunotherapies.

P682

Enhancing the engraftment of *ex-vivo* manipulated HSPCs by CRISPR-based epigenome editing

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Autologous transplantation of gene-corrected hematopoietic stem and progenitor cells (HSPCs) can effectively replenish defective blood cell production caused by congenital or acquired disorders, such as primary immunodeficiency diseases or hematopoietic malignancies. During autologous hematopoietic stem cell transplantation, the HSPCs are harvested from the bone marrow (BM) or mobilized and collected from the peripheral blood of the patient and enriched by isolation of the $CD34^+$ fraction that contains only few long-term repopulating multipotent HSCs and far more committed progenitor cells. HSPCs are then genetically corrected *in vitro* through viral gene therapy or nuclease-mediated gene editing and re-infused back to the patient by intravenous transplantation. Effective treatment requires the successful homing and engraftment of transplanted HSPCs into the BM niche, where they undergo self-renewal and establish a population of genetically modified cells that pass a correct copy of the gene to the blood cell progeny. Despite the excellent levels of gene correction currently attainable *in vitro*, the translational potential of this approach has been hampered by a poor engraftment of engineered HSPCs *in vivo* after transplant. Most likely, this is caused by some cell intrinsic defect such as reduced homing capacity or the loss of HSPCs repopulating potential due to the *ex vivo* manipulation. This represents an impellent hurdle to overcome to achieve the full potential of HSPC-based cellular therapy. To enhance the efficacy of HSPCs transplantation, we devised an epigenome platform for the transient upregulation of chemokines and integrins involved in stem cell homing and engraftment. We used a highly specific CRISPR activation system composed by a catalytically dead Cas9 protein fused to the tripartite transcriptional activator VPR (dCas9-VPR) and sgRNAs specifically designed to recruit dCas9-VPR to the promoter region of the target genes and activate their transcription. The time-course analysis of gene expression revealed a peak of gene activation after 24h and the restoration to the baseline expression levels after 96h, demonstrating that this platform is compatible with the kinetics of HSPCs homing and engraftment after transplant thus avoiding the potential negative effects that could be caused by persistent perturbation of gene expression in HSPCs. We also tested the efficiency of this system in providing a homing and engraftment advantage by *in vitro* migration assay of HSPCs gene-edited with a CRISPR/Cas9 platform for the treatment of Wiskott-Aldrich Syndrome developed in our lab. By integrating this epigenome platform for the engraftment enhancers upregulation with already established gene-editing platforms for gene correction, it will be possible to ensure high levels of engraftment of corrected HSPCs in the BM providing a bench-to-bedside translation of gene editing technologies to treat a wide range of blood disorders.

P683

Enhancing clarity and safety of gene editing with MEGA: A comprehensive digital PCR-based analysis of chromosomal aberrations in nuclease-edited primary cells

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Designer nuclease gene editing holds great promise for therapeutic applications; however, there are safety risks that hinder clinical translation. Understanding the extent of unintended chromosomal aberrations arising from nuclease-based editing, therefore, is crucial for its advancement. Current analytic techniques and innovative sequencing strategies provide us with valuable knowledge about the frequency, location, and type of chromosomal aberrations, aiding in the prediction of genotoxic potential. Nonetheless, these approaches have limitations, including high costs, time-consuming procedures, and infer a narrow, and sometimes biased, set of information. We address these challenges with Multipurpose Editing and Genotoxicity Assessment (MEGA), a novel diagnostic set of multiplexed assays utilising digital PCR (dPCR) for the comprehensive characterisation of unintended mutations at both on- and off-target sites. MEGA offers valuable information on genome integrity, targeted integration, and extrachromosomal non-integrated vectors. To demonstrate the applicability of MEGA, we utilised multiple designer nuclease platforms combined with adeno-associated virus (AAV) transduction to edit therapeutic and aberration characterised gene targets in human-derived haematopoietic stem and progenitor cells (HSPCs) and T cells. After editing with Cas9, Cas12, or Transcription activator-like effector nucleases (TALENs) in clinically relevant cells, MEGA can effectively quantify the frequency of indels, large deletions, loss of the chromosomal arm, targeted integration, and the remaining unedited cells within the total population. Moreover, longitudinal analyses reveal the stability of subpopulations with varying aberrations over extended periods of time, allowing for new insights into DNA cleavage kinetics, repair and mutation formation rates, and targeted integration dynamics. Furthermore, we observed a reduced frequency of indel formation during AAV-mediated targeted knock-in of GFP when compared to nuclease-only editing and prolonged retention of episomal AAV material in cells beyond the detection of GFP expression via flow cytometry. We also assessed the detection sensitivity of various anticipated mutations within the overall allelic population by quantifying serially diluted synthesised mutation cassettes. In summary, MEGA fills a critical niche in the post-editing analysis toolbox by offering a rapid and specific overview of genome integrity with contemporary gene-editing techniques. By providing comprehensive and efficient characterisation of unintended aberrations, MEGA contributes to the safety and effective translation of designer nuclease gene editing into clinical applications.

P684

Attenuation of SASP through sEV biogenesis interruption

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Cells have the capacity to modulate the microenvironment through secreted molecules and factors like cytokines, chemokines, small extracellular vesicles (sEV)... Senescent cells are characterized by a specific secretome phenotype known as SASP, an inhibition on the cell cycle, and an increased β-galactosidase activity. SASP leads the microenvironment to a more pro-inflammatory state and induces senescence in the neighbouring cells, triggering with time age-related diseases. To study this paracrine senescence transmission, we focused on the role of sEV. In this work, we pretend to find a proteomic target for the SASP mediated by sEV to reveal pathways associated with the senescence transmission. We knocked-down the expression of *RAB27A* and *RELA*, proteins implicated in sEV biogenesis and in paracrine senescence respectively, in mesenchymal stem cells from umbilical stroma cord. It was accomplished to reduce the paracrine senescence transmission by sEV in the knock-down cells after the addition of vesicles from senescent cells, validated by β-galactosidase activity and proliferation assays. We also performed a shotgun proteomic study of the recipient cells, identifying several proteins statistically significant dysregulated involved in the Golgi traffic and network. In conclusion, the silencing of *RAB27A* prevents the paracrine senescence transmission and a few proteins could be future targets for the development of senomorphic drugs.

P685

High frequency HDR gene editing of γ-globin promoters produces therapeutic levels of fetal hemoglobin *in-vivo*

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Despite being the earliest known molecular diseases, β-hemoglobinopathies remains incurable and widespread. Altered γ-globin silencing in naturally existing HPFH condition emphasize the role of genetic variants to establish durable fetal hemoglobin (HbF) re-activation. Non-deletional HPFH furnishes elevated HbF levels in adults with minimal genomic interference, since they majorly constitute point mutations in the homologous γ-globin (*HBG1* and *HBG2*) promoters. Here, we deployed the CRISPR/Cas9 homology directed repair (HDR) using ssODN donor template to inscribe two HbF inducing conversions, -175T>C and -158C>T at the *HBG1/HBG2* promoters, for the therapeutic reactivation of HbF. Precise incorporation and incomplete editing tracts generated an overall beneficial modification frequency of 30% in therapeutically relevant hematopoietic stem and progenitor cells (HSPCs). Our data indicate that

asymmetric, non-target ssODN mediates elevated levels of HDR conversions in HSPCs, resulting in 60% F+ve cell fraction in erythroid cells differentiated from edited HSPCs. Furthermore, in our screen testing across small molecule enhancers, DNA-PK inhibitor AZD-7648 outperformed others in improving HDR gene editing in HSPCs. Minimizing the interference of NHEJ repair using AZD-7648 post electroporation, amplified the precise HDR correction frequency to ~65% *in-vitro*. Notably, the xenotransplantation of enhancer edited HSPCs into NBSGW mice revealed the retention of up to 33% HDR edited cells within the bone marrow harvested at 16th week post-transplant. The engraftment of -175T>C, -158C>T dual genetic variant HSPCs also contributed up to 45.7% F+ve cells *in-vivo*. Our study suggests that careful screening of ssODN design, dosage, culture and editing conditions, enables the generation of high frequency, durable HDR editing in HSPCs.

P686

A reporter transgene to screen serine integrases for gene editing in human T cells

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Immunotherapy with gene-edited T cells has emerged as a powerful approach for the treatment of various malignancies. The primary manufacturing method of engineered T cells, such as chimeric antigen receptor (CAR) T cells, relies on lentiviral transduction. While this approach enables efficient introduction of DNA constructs into patient-derived T cells, it comes with significant costs and poses certain challenges, such as the risk of unintended gene disruption or activation of proto-oncogenes due to semi-random integration of viral vectors. As an alternative, gene editing techniques are being explored, with current strategies using electroporation with programmable nucleases, such as CRISPR/Cas9, for the induction of homology-directed DNA repair leading to targeted integration of DNA inserts. However, these methods often suffer from low efficiency and limitations regarding DNA cargo size, thus compromising the quality and therapeutic potential of engineered T cells.

To overcome the aforementioned challenges, we propose the utilization of site-specific integrases. By leveraging the inherent ability of certain integrases – in particular large serine integrases – to mediate site-specific recombination, DNA cargo can be introduced into the genome via sequence-defined landing sites. This should enable the precise integration of large DNA cargo – including multiple gene inserts – at prespecified sites.

Recently, multiple groups have performed large-scale discovery of serine recombinases to identify novel enzymes suitable for genome engineering in mammalian cells. However, to date, only a few of these serine integrases have been evaluated for their efficacy for site-directed recombination in primary human T cells.

To assess and compare the efficiency of various integrases in human T cells, we designed a reporter transgene for integration in the endogenous *TRAC* locus. The reporter consists of an artificial intron that contains an array of recognition sequences (landing pads) for different serine integrases, followed by an EGFP reporter gene. We successfully generated a T cell line containing the artificial intron and expressing the downstream GFP. To test recombination in our reporter, we designed an RFP expressing vector featuring a corresponding array of recombination sites for

integration into the artificial intron. This array in the integration vector is followed by a splice acceptor, terminating the artificial intron upon successful recombination and facilitating the expression of RFP without an exogenous promoter. Analyzing EGFP and RFP expression after transfecting T cells containing the landing pads with the integration vector and the respective integrases, allows us to compare integration efficiencies in order to identify the most promising site-specific serine integrase. This setup enables parallel screening of different integrases and the artificial intron might minimize potential bias due to the order of the landing pad sites, thereby ensuring comparability. In the future, we plan to combine nuclease-assisted gene editing for the insertion of the landing site into the genome and subsequent site-specific recombination to incorporate large vectors via serine integrases in a single transfection. The integration of larger multicistronic constructs through this integrase-based approach should open up new possibilities for engineered T cells with enhanced functionalities, thereby driving further advancements in T cell-based immunotherapy.

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Topical *In Situ* Gene Therapy for the Treatment of Autosomal Recessive Congenital Ichthyosis Patients

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Autosomal Recessive Congenital Ichthyosis (ARCI) encompasses a group of severe skin conditions characterized by abnormal keratinization processes. These disorders arise due to mutations in several genes, with one of the most affected genes being TGM1, which encodes the enzyme Transglutaminase-1 (TGase-1). A specific mutation, c.877-2A>G in TGM1, is present in approximately one-third of all TGM1 mutation alleles, resulting in a premature stop codon in the human genome. TGase-1 is a critical enzyme involved in the crosslinking of the stratum corneum, the outermost barrier of the skin. Patients with ARCI typically exhibit dry, scaly skin areas with impaired skin barrier function, leading to high transepidermal water loss and increased susceptibility to infections. These manifestations can be particularly severe and life-threatening, especially in neonates. Current treatments for ARCI aim to improve the quality of life by removing loose skin and frequent application of moisturizers. However, these treatments do not address the underlying cause of the disease.

Recent advancements in gene-editing techniques, particularly base editing, offer potential cures for single-gene mutations causing ARCI. Nevertheless, the delivery of gene editing tools to the skin remains challenging due to the skin's restrictive barrier, even in diseased states, with most current regimens limited to symptomatic treatments rather than cures. To bridge this treatment gap, we established human *in vitro* skin models and developed an *in situ* gene therapy approach involving physical modulation of the skin barrier through laser treatment, lipid nanoparticle (LNP)-mediated drug delivery, and CRISPR-Cas9-based base editing technology. In order to address the TGM1 c.877-2A>G mutation, we employed NG-BE4max, a cytosine base editor that explicitly targets the non-coding strand. This approach facilitated a base exchange from the mutant G to the wild-type A on our target strand, effectively correcting the TGM1 c.877-2A>G mutation. We assessed the safety and efficacy of our approach in human skin models and achieved *in situ* editing rates of ≥10% in excised and reconstructed human skin without inducing pro-inflammatory cytokine release. In ARCI patient cells, our gene-editing tool, specifically designed for the patient's mutation, led to approximately 5% base editing rates, indicating efficient

correction of disease-causing mutations. Notably, emerging evidence suggests that correcting 5-10% of disease-causing mutations may be sufficient to alleviate the symptoms of severe genetic diseases such as genodermatoses. In conclusion, our approach aims to correct disease-causing mutations *in situ*, offering effective treatment and a potential cure for rare skin diseases. Further studies and clinical trials are warranted to validate the safety and long-term efficacy of this gene editing strategy in a clinical setting.

P689

Target sequence-independent detection of CRISPR-Cas off-target effects by universal CAST-Seq

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Genome editing can be associated with genotoxicity, such as mutagenesis at off-target sites and chromosomal translocations. This risk must be carefully evaluated before gene-edited products can be used clinically. We recently described CAST-Seq, a diagnostic assay that detects CRISPR-Cas-induced chromosomal aberrations in clinically applied cells in a genome-wide manner. The method is based on the detection of chromosomal rearrangements resulting from the simultaneous cleavage of the on-target and an off-target site. While CAST-Seq is able to identify CRISPR-Cas nuclease-induced chromosomal aberrations with high sensitivity, the sensitivity to detect off-target activity of base editors or prime editors is lower because these editing platforms do not rely on the formation of DNA double-strand breaks. In addition, in rare cases, designing effective CAST-Seq primers for a specific target site in GC-rich regions of the genome can be tedious. To overcome these limitations, we developed universal CAST-Seq (U-CAST), a method to detect genome-wide off-target activities of any CRISPR-based editing platform without the need for primer optimization. U-CAST is based on the fact that any DNA double-strand break, inter alia one introduced by a highly specific CRISPR-Cas12a nuclease in the *CSF2RA* locus, can serve as an "anchor" to detect off-target events triggered by any genome/base/prime editor. After adapting the bioinformatics pipeline, we provide evidence that U-CAST is able to nominate off-target sites and identify chromosomal rearrangements triggered by different CRISPR-based editors. Performing U-CAST with CRISPR-Cas9 nucleases targeting reference genes, such as *VEGFA*, confirmed that the results obtained with U-CAST are comparable to those obtained with conventional CAST-Seq with minimal compromises in terms of sensitivity: some ultrarare translocation events were missed. Because the method can be performed with already established U-CAST primers, U-CAST saves time and cost, and is particularly well suited for screening designer nucleases and base editors in early development.

P690

The use of hfCas12Max platform in gene therapy and cell therapy

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In recent years, the search of new class 2 CRISPR-Cas systems has led to the discovery of Cas12i. Althought Cas12i has the potential to serve as genome-editing tool, the editing efficiency and specificity need to be improved before effecitive application.The hfCas12Max is an engineered Cas12i recognizing 5'-NTN PAM with high editing activity and specificity. Compared to Cas9 and Cas12a, hfCas12Max has a smaller size and is more suitable for a single adeno-associated viral (AAV)-based delivery. Here, we tested the editing efficiency of hfCas12Max both *in vivo* and *in vitro* using different delivery methods, such as AAV, lipid nanoparticle (LNP), and ribonucleoprotein (RNP). AAV is the most frequently used gene-therapy delivery vehicle due to it effective and safe track record and wide range of tissue targeting, including skeletal and cardiac muscle. After systemic delivery of a single all-in-one AAV vector containing hfCas12Max and a guide RNA (gRNA) targeting the splice donor site of human exon 51 to the Duchenne muscular dystrophy (DMD) mouse model, the dystrophin expression was efficiently restored, and histopathology and grip strength were ameliorated. In further exploration to neurodegenerative diseases including amyotrophic lateral sclerosis (ALS) and Huntington's disease (HD), hfCas12Max-mediated knockdown efficiency of toxic proteins exceeded 80%. Moreover, targeting the gene cluster with a single gRNA, hfCas12Max restored over 80% of Ube3a protein expression in Angelman syndrome mice. Through RNP delivering hfCas12Max targeting *TRAC* and *B2M* in primary human T cells, we detected ~90% editing efficiency at both sites in CD3+ T cells, with the cell viability remaining at ~80%. Furthermore, by targeting the hepatitis B cccDNA of mouse liver and primary human hepatocytes (PHH) using LNP with hfCas12Max mRNA and gRNAs, we detected a dramatic decrease in cccDNA. In silico and genome-wide off-target analyses revealed the high specificity of hfCas12Max with the corresponding gRNAs. Together, our findings demonstrate that hfCas12Max has robust editing activity and high specificity, suggesting it as a promising tool for disease modeling as well as safer and more efficient treatments in gene therapy and cell therapy.

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RNA base editing therapy for *ABCA4*-associated Stargardt disease type 1

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Stargardt disease (STGD) is an inherited, autosomal-recessive retinal disease that typically presents visual impairment and blindness in the first two decases of life, affecting 1 in 8000-10,000 individuals. Stargardt disease type 1 (STGD1) is the most common form of inherited macular degeneration, caused by the bi-allelic mutations in the *ABCA4* gene encoding an ATP binding cassette protein family member 4 (ABCA4) which is essential to the visual cycle and is located specifically in the outer segments of the photoreceptor cells. The most frequent variant

c.5882G>A, p.G1961E, a G-to-A point mutation, affects approximately 15% of the STGD patients. Patients present with bilateral central vision loss, including dyschromatopsia and central scotomata, with macular atrophy and yellow-white flecks at the level of the retinal pigment epithelium (RPE) at the posterior pole. Although there are currently no curative treatments and STGD1 gene replacement therapy clinical trials are ongoing, the large 6.8kb size of *ABCA4* gene presents a major challenge due to the carrying capacity limitation of an adeno-associated virus (AAV). Here, we developed a site-directed RNA base-editing therapeutic strategy using the gRNA targeting specific mutation sites and the mini-dCas13X adenine base editor (mxABE) which is composed of mini-dCas13X protein and ADARdd protein to correct the G1961E mutation at the RNA level to restore the expression of functional ABCA4 protein. To target the c.5882G>A mutation, we tested different guide RNAs (gRNAs) in HEK293T cells carrying the mutation in *ABCA4* and achieved up to 40% editing efficiency. Delivered by a single AAV, mxABE with gRNA targeting ABCA4 (mxABE-ABCA4) showed an editing efficiency of up to 17.5% (5.0E+9/eye) in homozygous humanized *ABCA4*^{G1961E} mouse model. We further improved the system by replacing the promoter, adding nuclear localization sequences, and introducing a short nucleotide sequence (exin21) to enhance mRNA stability, resulting in significant improvements of *in vivo* editing efficiency (up to 40%) but no obvious phenotype changes. The benefit of RNA editing is that it does not directly alter the native DNA and that any edits are transient for the lifetime of the mRNA molecule. Therefore, we demonstrated that the CRISPR-Cas13-derived RNA base editing system is a feasible and safer therapeutic approach for the treatment of STGD1 as we continue to further optimize this CRISPR-based strategy.

P692

Cell-targeted gene modification by delivery of CRISPR/Cas9 RNPs in pseudotyped lentivirus-derived nanoparticles

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Development of delivery vehicles allowing targeted delivery and time-restricted activity of genome editing tool kits is an important goal towards the implementation of safe CRISPR/Cas9-based gene editing therapies. Here, by modulating the pseudotype of engineered lentivirus-derived nanoparticles (LVNPs), we demonstrate efficient cell-targeted Cas9/sgRNA RNP delivery using LVNPs, leading to gene modification in a subset of cells in mixed cell populations. For LVNPs pseudotyped with SARS-CoV-2 spike protein, indel formation was evident in >95% of ACE2-positive cells, but not in cells lacking ACE2. Additionally, we found that pseudotyping of LVNPs with nipah virus glycoproteins resulted in 70% *B2M* knockout in Ephrin-B2/B3-positive cells without affecting cells without Ephrin-B2/B3 expression. LVNPs pseudotyped with Edmonston strain measles virus glycoproteins (MV-H/F) delivered Cas9/sgRNA RNPs to CD46-positive cells with and without additional expression of SLAM (CD150). However, an engineered MV-H/Y481N SLAM-specific measles virus pseudotype (MV-H/F-SLAM) efficiently targeted LVNPs to SLAM-positive cells (indel rates >60%) in a mixed population of cells containing also SLAM-negative cells. Lentiviral vectors pseudotyped with MV-H/F-SLAM efficiently transduced 80% of activated primary B cells that were cultured on CD40L-expressing MS-5 feeder cells and stimulated with IL-4 and IL-21. LVNPs pseudotyped with MV-H/F- and MV-H/F-SLAM resulted in indel rates of >80% and >60%, respectively, in activated primary B

cells, whereas pseudotyping of LVNPs with VSV-G did not result in detectable indel rates. Collectively, our findings demonstrate the modularity of LVNP-directed delivery of ready-to-function Cas9/sgRNA complexes, supporting engineering of LVNPs that induce effective indel formation in a subpopulation of cells defined by the presence of receptors matching a specific pseudotype.

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A universal CRISPR/Cas9 gene editing approach as a definitive cure for DOCK8 immunodeficiency syndrome

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Inborn errors of immunity (IEIs) encompass a diverse range of genetic disorders that exhibit immune dysregulation and render individuals highly vulnerable to infections and other immune-related autoimmunity. Among these disorders, DOCK8 deficiency, a combined immunodeficiency, typically manifests during the first decade of life and results in recurrent viral and bacterial infections, atopic diseases, and malignancies, leading to significant morbidity and mortality. Although allogeneic hematopoietic stem cell transplantation is the recommended therapeutic approach, limitations such as lack of HLA-matched donors and the risk of graft versus host disease emphasize the need for alternative treatments.

Here, we pursue the development of a gene therapy for DOCK8 deficiency by employing CRISPR/Cas9-mediated gene correction. The optimal therapeutic approach involves an *ex vivo* site-specific correction of the disease-causing mutation in the patient's own hematopoietic stem cells (HSCs), hence enabling reconstitution of the immune system. To accommodate the profound diversity in genetic variants associated with DOCK8 deficiency we devise a strategy that integrates a DOCK8 cDNA encompassing exons 25 to 48 into the endogenous *DOCK8* exon 25 This strategy is applicable to >50% of the identified pathogenic disease-causing variants. We employ electroporation-mediated delivery of sgRNAs targeting exon 1 and 25 of *DOCK8* complexed to Cas9 ribonucleoprotein (RNP). Six sgRNAs were systematically screened resulting in efficient gene editing of the *DOCK8* gene with up to 95% target-specific indels in the K562 immortalized lymphoblast cell line. Subsequently, to test targeted integration of new DNA at the DOCK8 locus, AAV6 donor templates with 400bp homology arms and an SFFV-GFP cassette were designed for the best sgRNA. Electroporation of Cas9 RNP followed by DNA donor delivery by AAV6 transduction gave rise to *DOCK8* targeted integration in 50% of K562 cells and 40% of healthy human hematopoietic stem and progenitor cells (HSPCs). Additionally, long-term repopulation capability of gene edited HSPCs was investigated through the analysis of humanized mice (n=6) at 16 weeks post transplantation of immunodeficient NOD scid gamma mice. These studies showed efficient engraftment with 14% humanization in the bone marrow and GFP expression in 5% of the human graft compared to 55% in the input HSPCs. Finally, a DNA donor construct containing the cDNA of DOCK8 exons 25-48 was generated, utilizing the maximum capacity of a single AAV vector. Initial validation of cDNA integration in the THP-1 cell line was performed by semiquantitative in-out PCR and quantitative ddPCR. Future studies will involve establishing a THP-1 DOCK8 knockout model for a comprehensive evaluation of the capability of the strategy to restore DOCK8 function and actin- cytoskeleton dynamics.

In summary, this research project utilizes CRISPR/Cas9 to specifically target the DOCK8 gene, aiming to develop a potential cure for DOCK8 deficiency. The preliminary findings support the feasibility of DOCK8 gene editing and highlight the potential for future therapeutic interventions. Further research is required to optimize and validate our gene correction strategy and to ultimately provide hope to patients suffering from this debilitating disorder.

P694

Computationally defined genomic safe harbour loci enable stable, as well as inducible, transgene expression in human cells

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Stable expression of transgenes is essential in both therapeutic and research applications. Traditionally, transgene integration is done via viral vectors in a semi-random fashion, this carries the risks of transgene silencing and disruption of endogenous genes. Targeted integration of transgenes into genomic safe harbour (GSH) loci in the human genome has been proposed as safer alternative. Although several integration sites have been characterised for transgene integration in the literature, most of these do not meet criteria set out for a GSH. Here, we conducted a computational analysis using publicly available data to identify 25 unique putative GSH loci that reside in active chromosomal compartments. We validated three candidate GSH sites *in vitro* by integrating a constitutively expressing landing-pad cassette into H1 and H9 human embryonic stem cells (hESCs) as well as human induced pluripotent cells (hiPSCs) using CRISPR-Cas9. Minimal disruption of the native transcriptome was confirmed by qPCR and RNA sequencing of the established cell lines. To validate stable transgene expression, we swapped in a green fluorescent protein transgene into the landing-pad sites in our H1 and H9 clonal lines. High transgene expression was confirmed in hESCs over 15 passages via flow cytometry and immunofluorescence. We differentiated the targeted hESC into all three germ lineages and confirmed persistent transgene expression with immunofluorescence and high content imaging using relevant marker proteins to each respective cell lineage. Furthermore, we demonstrate inducible transgene expression from our GSH by integrating a TetO-driven expression construct into one of the GSH landing pad expression cell lines in both H1 and H9 hESCs. The generated hESC and iPSC GSH landing pad expression cell lines offer an efficient way of targeted and controlled expression of genes of interest for many research applications. In the future, the described GSH may enable expression of therapeutic genes in e.g., iPSC-based cell therapy.

P695

IDLV as template delivery platform for *RAG1* gene editing

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Regulated *recombination activating gene 1* (*RAG1*) expression is crucial for the V(D)J recombination process that takes place during B and T cell differentiation, and mutations in *RAG1* are associated with several heterogeneous immunological disorders. Using a knock-out/knock-in gene editing (GE) strategy based on CRISPR/Cas9 and adeno-associated vector serotype 6 (AAV6) delivery of the corrective template, we have recently demonstrated correction of *RAG1* expression and activity in patient-derived hematopoietic stem/progenitor cells (HSPCs). Given the previously published favorable safety profile of integrase-defective lentiviral (IDLV) over AAV6 for the GE process, we transitioned to IDLV as alternative template delivery system for homologous directed repair (HDR) editing. We initially tested the IDLV corrective donor templates in NALM6.Rag1 knock-out (KO) cell line to assess RAG1 rescue. Cells were subcloned by single cell sorting and screened for editing, and levels of correction were measured by testing *RAG1* gene expression and function. Here we obtained good levels of *RAG1* expression and recombination activity. Thus, we decided to evaluate the impact of IDLV platform on human HSPCs. We tested different doses, timings and rounds of IDLV transduction in mobilised peripheral blood (mPB) HSPCs from healthy donors in order to select the best performing protocol for IDLV, which was then directly compared with the AAV6 based one. Both platforms showed high and comparable levels of HDR in mPB-HSPCs. Importantly, as recently shown, we confirmed higher editing efficiency in long-term repopulating LT-HSPCs with IDLV than AAV6. Moreover, cells treated with AAV6 or IDLV platforms showed comparable HSPC composition. Upon xenotransplantation, engraftment in mice receiving HSPCs edited with the IDLV template was more stable than those edited with the AAV6 one. Taken together these data demonstrate that the IDLV can be successfully used as an alternative to AAV6 for *RAG1* GE, mitigating the genotoxic risks of the GE procedure, and facilitating clinical translation of the *RAG1* GE strategy.

P696

Efficiency of multiplexing guide RNAs for multi-gene CRISPR/Cas9-based genomic modulation

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Developing therapeutic strategies to simultaneously modify multiple genomic loci may be required to enable treatment of conditions with complex etiology using genetic medicines. Targeting multiple genomic loci simultaneously can be achieved using CRISPR/Cas9-based gene modulation technologies that deliver multiple guide RNAs (gRNAs) in a single AAV viral vector. One way to

drive the expression of multiple gRNAs in a single cell is to use a unique PolIII promoter for each gRNA. However, due to the size limitations of AAV viral vector packaging, delivering multiple gRNAs each with their own unique promoter limits the number of genomic loci that can be simultaneously targeted. An alternative strategy is to use a single promoter to drive an array of gRNAs, each of which is separated by endogenous transfer RNA (tRNA) sequences. This strategy harnesses endogenous RNaseP and RNaseZ activity to remove the tRNAs, thereby releasing the gRNAs for Cas9-mediated gene expression modulation.

Here we demonstrate the feasibility and efficiency of delivering an array of gRNAs interspersed with tRNA sequences *in vitro*, compared to delivering gRNAs driven by multiple unique promoters. We present results on efficiency of gene expression modulation with multiplexed gRNAs at the RNA level as well as next-generation sequencing analyses to measure the efficiency of gRNA processing from the multiplexed transcript using various models. We find that level of target gene modulation varies depending on the position of the gRNA in the multiplexed array, the number of gRNA copies present, and the identity of the targeted sequence. By simultaneously modulating multiple loci using a single AAV viral vector, this work further expands the capability of AAV-based genetic medicine platforms to treat a variety of conditions.

P697

Multiplex gene repair by mRNA-mediated base editing in compound heterozygous muscular dystrophy

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Autosomal recessive limb-girdle muscular dystrophy subtype 2D (LGMD 2D) is a heterogeneous progressive disorder manifesting muscle wastage without any treatment. Homozygous or compound heterozygous variants in the α-*SGCA* gene are known genetic causes of this condition. Base editor (BE) mediated single-nucleotide conversions without double-stranded DNA breaks (DSBs) are considered relatively safe for therapeutic applications. We recently introduced CRISPR/Cas9 based mRNA molecules into primary human muscle stem cells (MuSCs) that resulted in >90% efficient genome editing. Moreover, we showed that gene-edited human MuSC can regenerate muscle *in vivo* in xenografts, making them suitable for application in autologous cell replacement therapies for LGMD. In case of compound heterozygous mutations, it is hypothetical whether repairing one mutation alone would suffice to provide adequate gene-dosage. Therefore, we evaluated whether simultaneous multiplex-editing is possible in primary human MuSC. We obtained pure MuSC populations from two patients carrying compound heterozygous mutations in the *SGCA* gene. *SGCA* c.739G>A is a frequent founder mutation common to both families while the second mutation differed between the families. These mutations were candidates for repair by adenine base editing (ABE) that enables the seamless conversion of A:T to G:C base pairs in a defined editing window at the target site determined by the supplied single guide RNA (sgRNA). We delivered mRNA encoding the first (ABE7.10) and newer (ABE8e) generation ABE with a classical NGG PAM requirement, as well as a variant with relaxed PAM specificity, ABE8e-SpRY, in combination with suitable sgRNAs targeting the mutations to patient MuSC. Editing of each mutation individually was possible. We then selected the most promising single ABE and dual sgRNA combinations for precise and simultaneous repair of the compound heterozygous mutation pairs. Multiplex editing led to high nucleotide conversion rates at each target site and resulted in efficient double repair of both mutations in MuSC from both patients. Myogenic and proliferative

properties of edited MuSC were preserved. MuSC-derived myotubes from both patients following multiplex base editing shows a robust rescue of α-SGCA protein. We report here highly efficient and functional multiplex gene repair in primary patient MuSC, with immediate implications for autologous transplantation therapies in disorders that previously lacked any therapeutic perspective.

P698

A comprehensive off-target analysis pipeline for Prime Editing reveals a favorable safety profile

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Gene editing can restore normal function to damaged genes; however, the application of gene editing to the clinic is limited by the potential genotoxicity of off-target events. Compared to gene editing approaches that rely on the generation of double strand breaks or non-sequence specific base altering activities, Prime Editing may have a fundamental safety advantage as it only nicks one strand of DNA and any edits it makes require additional sequence homology and are subject to specificity checks by the cell's DNA repair machinery. Building on concepts or approaches designed for CRISPR off-target analysis, we have developed a suite of assays to identify potential off-target events resulting from Prime Editing. We will describe and show results from these assays in the context of model targets and with data from one of our late-stage preclinical gene editing programs. Preliminary analysis of Prime Edited cells from the preclinical program has identified neither off-target indels nor chromosomal alterations. Collectively, our results show a comprehensive pipeline to identify and evaluate potential off-target events and support an excellent safety profile for Prime Editing technology.

P699

NIST Genome Editing Consortium interlab study to evaluate the performance of DNA detection technologies in use for confirming genome editing

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Moving genome editing technologies into production and medical practice for cell & gene therapy requires robust quantitative assays, with associated controls and data tools. The U.S. National Institute of Standards and Technology (NIST) Genome Editing Consortium (GEC) convenes experts across academia, industry, and government to collaboratively address precompetitive genome editing measurements and standards needed to increase confidence in evaluating genome editing and utilizing these technologies in research and commercial products. NIST GEC led efforts have

resulted in the first international standard for genome editing terminology: International Standards Organization- ISO 5058-1:2021 Biotechnology — Genome editing — Part 1: Vocabulary

The NIST GEC has completed a first of its kind interlab study with organizations in the genome editing field to understand the performance of DNA detection technologies in use for confirming on- and off-target genome editing. NIST generated DNA- or cell-based control samples, qualified by ddPCR and NGS, to evaluate the accuracy of the study participants' reporting of the size, sequence, and frequency of DNA variants. DNA variant benchmarks within control samples ranged in size from single nucleotide variants (SNV) to insertions and deletions tens of kilobases long. Variants were present within the interlab study control samples at all of 5 primary frequency bins: 0%, >30%, 5 - 10%, 0.5 -2%, and 0.1 - 0.25%; with two optional samples of ~0.01% and ~0.001%. Participants were provided the 5 required samples and a core list of 39 genomic positions of interest to be analyzed by any type of DNA detection process being utilized by the interlab participant. Interlab participants were blinded as to variant sequence and variant frequency within each sample and at each position of interest.

14 Interlab participants returned their results along with: metadata describing the assay and data analysis approach, raw data files, and a list of variants detected- complete with variant positions, sequences, and frequencies. One to two technologies were assessed per participant, with 1-4 replicates per technology. Technologies assessed include: bulk DNA short read NGS, bulk DNA long read NGS, single cell targeted NGS, genome wide DNA imaging, targeted DNA probe imaging, and capillary electrophoresis fragment analysis. NIST evaluated the results from individual technologies for accuracy of variant detection and frequency prediction. While no one technology had the capability to detect all of the variants at all of the frequencies and sizes, combined results from all of the technologies evaluated, confirmed the ability to measure variant sizes from SNV to >100kb, and a subset of variants down to 0.001%. Trends in performance were identified as well as instances where variant signal was present but not reported by the bioinformatics process.

The NIST GEC is working towards a database where all datasets, metadata, and results could be made publicly accessible. Further in-progress NIST GEC work includes development of metadata norms and clonal engineered cell lines to be used as DNA/cell based controls supporting greater assay confidence for the genome editing community and development of genome edited cell based therapies.

P700

Correcting the *CFTR* 1717-1G>A splicing mutation by CRISPR-Cas strategies

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The substitution known as 1717-1G>A is a commonly observed *CFTR* Class 1 mutation, affecting around 1% of individuals with cystic fibrosis (CF) according to the CFTR2 database. This specific mutation involves the conversion of a G to an A in intron 11, leading to disruption of a conserved AG dinucleotide at the 3' end acceptor splice site. Consequently, exon 12 is skipped due to the recognition of a newly formed cryptic splice site. The alternative splicing products resulting from this mutation generate a premature stop codon, preventing the synthesis of the CFTR protein. The objective of this study is to develop cutting-edge genome editing approaches to correct the 1717-1G>A mutation, based on prime and base editing technologies.

To establish the CRISPR-Cas strategy, a cellular model of the 1717-1G>A mutation was generated using HEK293 cells. Through plasmid transfection, we explored prime editing technology, which resulted in an efficient correction rate of over 40%. Moreover, the splicing pattern and membrane localization in the 1717-1G>A HEK293 model were significantly improved. However, the prime editing approach did not work efficiently in the endogenous *CFTR* locus. Therefore, an alternative strategy utilizing a nuclease-based prime editor was investigated, yielding editing efficiencies above 20%, although significant InDels (insertions and deletions) were generated.
To overcome the limitation of the prime editing approach we turned to a base editing strategy obtaining over 30% A>G correction using SpRY-ABE9, with minimal bystander editing and similar degree of CFTR recovery indicated by proper protein localization at the cell membrane.
In summary, our data show that various CRISPR-Cas approaches based on prime and base editing can be utilized to repair the 1717-1G>A mutation. Notably, the recently developed ABE9 has turned out to be a promising strategy that balances editing efficiency and unwanted editing events. Ongoing efforts are being made to further optimize the base editing approach for correcting the mutation in patient-derived epithelial cells.

P701

Improved CRISPR gene editing with dbDNA as a knock-in template

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Recent advancements in non-viral gene editing technologies offer precise genome engineering at significantly reduced costs and biosafety risks compared to viruses. CRISPR is a programmable genome targeting system, which relies on a donor DNA as a template for homology-directed repair (HDR) to introduce exogenous sequences for cell and gene therapy. Therefore, as viruses are increasingly being replaced by safer and cheaper technologies, there is a growing need for reliable and effective vectors for targeted gene insertions. Plasmid DNA (pDNA), which has traditionally been used as the incumbent template for gene-length knock-ins, however, is associated with high toxicities, poor immunogenicity profiles and low HDR efficiencies, all of which has served to limit the success of non-viral gene therapy.

Touchlight's doggybone DNA (dbDNA™) is a fully synthetic, GMP-grade, covalently closed, linear vector, which directly addresses these challenges. The enzymatic manufacture eliminates bacterial sequences and produces a minimal vector of higher purity and lower immunogenicity compared to fermentation-derived DNA. Furthermore, the platform offers unparalleled scalability with reduced timelines overcoming pDNA manufacture bottlenecks.

dbDNA has been robustly evaluated as an HDR template in *ex vivo* CRISPR gene editing platforms internally and by gene therapy industry leaders and is shown to maintain significantly better cell viability compared to pDNA. Improved cell survival leads to accelerated recovery and expansion in culture to produce higher edited primary immune cell counts. With its reduced size, dbDNA has a copy-number advantage over pDNA, allowing improved CRIPSR knock-in rates at a lower template concentration, further minimising the DNA bioburden. Importantly, the improved toxicity and immunogenicity profiles lead to better and more reliable knock-in efficiencies across primary T cells isolated from different blood donors, reducing undesired donor-to-donor variability. This altogether makes dbDNA highly suitable for autologous ex vivo gene and cell therapies, where cell numbers are typically limited and genome editing outcomes inconsistent.

dbDNA's improved performance in cells, alongside Touchlight's robust and rapidly scalable manufacturing technology, offers a reliable and effective alternative to pDNA as a knock-in template for CRISPR and, thus, promises to advance non-viral gene therapy.

P702

In vivo genome base editing in a murine model of vanishing white matter alters the phenotype through multiple mechanisms

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Vanishing white matter (VWM) is a rare autosomal recessive neurologic disorder with a disease onset predominantly in early childhood. It is characterized by central nervous system (CNS) white matter degeneration, causing slowly progressive ataxia, spasticity and cognitive decline with stress-provoked episodes of rapid and major deterioration. VWM leads to progressive handicap and early death. It is caused by point mutations in the subunits of the eukaryotic translation initiation factor 2B (eIF2B). There are no effective therapies for this disease. Here, we assessed the potential of using CRISPR/Cas9-mediated adenine base editing to correct pathogenic mutations in the CNS of a mouse model of VWM (*Eif2b5*$^{R191H/R191H}$). Neurotropic adeno-associated viral vectors (AAVs) expressing adenine base editors (ABE) under the ubiquitous Cbh promoter were administered via intracerebroventricular injection at postnatal day 0.

Interestingly, the approach improved bodyweight and grip strength, however, other locomotor skills were mildly exacerbated. Moreover, slight locomotor deterioration was also observed in ABE-treated healthy control mice with a heterozygous mutation in *Eif2b5*. At 8 months after vector delivery, molecular analysis showed on average 32% (range 3.5 to 56%) correction of the pathogenic mutation (A to G) in brain tissue of VWM mice, but also moderate levels of nonsynonymous bystander edits (up to ~7.5%). The high efficiency of on-target correction resulted in amelioration of disease-specific phenotypic traits whilst the minor exacerbation of composite ataxia scores observed may be due to editing of bystander adenines around the target DNA sequence.

In conclusion, we achieved robust *in vivo* base editing of a pathogenic mutation in the *Eif2b5* gene in the CNS of VWM mice, but also undesired edits. This study showed the potential of base editing in CNS, but highlights limitations which could hamper disease-specific applications. Implementation of base-editors with higher specificity may exclude the observed side-effects and simultaneously enhance phenotype correction, which requires further investigation.

P703

Targeted genome editing of PMP22 TATA-box reduced PMP22 to therapeutic level in CMT1A patient derived iPSC-Schwann cells

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Charcot-Marie-Tooth disease 1A (CMT1A) is one of the most common hereditary demyelinating peripheral neuropathy driven by duplication mutation of 1.5Mb region in chromosome 17 including *PMP22* gene causing overexpression of PMP22 protein. Schwann cells, specialised myelin-forming glial cells in the peripheral nervous system (PNS) is thought to be primarily affected in CMT1A. We previously showed that CRISPR/Cas9-mediated targeted editing of TATA-box of PMP22 P1 promoter (Schwann cell-specific) can downregulate the level of PMP22 in a mouse model of CMT1A, harbouring multi copies of *PMP22*. To further prove our approach, here we utilised normal control and CMT1A-patient derived iPSCs to ascertain proof-of-concept in patient-specific manner. For this, we performed gene editing at the iPSC level and generated clones with precise PMP22 P1 promoter TATA box modifications. Subsequently these cell lines were differentiated into Schwann cells. Through gene expression and immunocytochemistry analyses we found significant increase in Schwann cell specific genes and confirmed Schwann cell marker expression. Importantly, PMP22 P1 promoter TATA-box edited CMT1A cell lines showed knockdown of PMP22 expression levels to normal control line. We also asked whether gene editing at the Schwann cell stage can result in knockdown of PMP22. For this, we screened different AAV serotypes and found AAV7m8 to be the most efficient transducer for iPSC derived Schwann cells. Treatment of CMT1A-patient derived iPSC-Schwann cells with AAV7m8 carrying CRISPR/Cas9 targeting TATA-box of PMP22 P1 promoter resulted in downregulation of PMP22. Throughout our study, we verified that CRISPR/Cas9-mediated editing of TATA-box of PMP22 P1 promoter can normalise PMP22 gene expression level in CMT1A-patient specific iPSC-Schwann cells and warrants further development of this approach for the treatment of CMT1A.

P704

Targeted CX3CR1 gene editing in HSPCs: Enhancing safety and transgene expression for effective paracrine cross-correction

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Lentivirus-based ex-vivo gene therapy for hematopoietic stem and progenitor cells (HSPCs) has demonstrated significant progress, but concerns regarding genotoxicity and insertional mutagenesis remain. To address these safety issues, gene editing (GE) tools offer a safer approach by enabling targeted insertion of expression cassettes. Our study aimed to achieve high transgene expression levels specifically in the myeloid lineage following HSPC editing to obtain a greater paracrine cross-correction without affecting HSPCs. We employed CRISPR-Cas9 to target the 4th

intron of CX3CR1nd designed various AAV6 donor DNA templates incorporating an SFFV promoter and a GFP reporter gene to optimize the knock-in process.

Surprisingly, the targeted insertion resulted in increased CX3CR1 levels specifically in HSPCs, with the most pronounced effect observed in the primitive CD34+CD4RA- population. Furthermore, the GFP reporter demonstrated a myeloid-skewed expression pattern, with the highest expression observed in CD34- committed myeloid populations during stem culture, and in M1 macrophages after differentiation. These findings indicate that the integrated cassette may be downregulated or silenced due to the epigenetic state of CX3CR1 during the early stages of HSPC differentiation.

Based on these results, we propose that CX3CR1 can serve as a safe harbour for targeted insertion of expression cassettes in HSPCs. This approach offers the advantage of a safer profile during stem stages while promoting higher transgene expression upon differentiation into promyelocytes. By leveraging the unique characteristics of CX3CR1, we can potentially enhance the safety and efficacy of ex-vivo gene editing strategies for HSPCs for paracrine cross-correction.

P705

How can we get more effective in implementing cell gene therapy in practice?

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Today 3,689 therapies are in development from the preclinical to pre-registration stage, of which 55 % are gene therapies. In the EU, there have been 20 approved cell & gene therapies (C&G), but only 12 are currently available for use. For rare disorders with approved C&G therapy, it's been reported that only 30 % of patients are diagnosed, and of those diagnosed, only 15 % receive C&G treatment, therefore, an important topic is how to ensure the timely availability of approved treatment and its incorporation in the standard therapeutic procedure. An increasing share of drug launches is coming from small start-ups with limited experience in launching and introducing the treatment.
Methods:

The key administration processes were mapped from diagnosis to finding the treatment. In our study, we attempted mapping of processes and identification of major differences versus other, non-cell gene treatments. Administrative processes for 5 different C&G therapies in 18 diagnostic and treatment centers in Europe were evaluated from diagnosis to treatment. The input was collected from 227 stakeholders responsible for the processes. An emphasis was placed on the fulfilment of standard legal and quality requirements. 125 patients were evaluated and C&G therapy was administered to 29 patients.

Results and Discussion: From diagnosis to initiation of the therapy, it took 6 to 18 months. The treatment approval took a minimum of 16 weeks from diagnosis. A total of 52 different processes were required from diagnosis to local treatment or cross-border treatment. Specific for C&G treatment, 21 processes (40,4%) requiring more than 100 additional hours to establish a treatment center, were identified. 31 processes (59,6%) related to the cross-border treatment (diagnosis assessment, treatment proposal, review, and implementation). The highest number of processes

related to the establishment and running of the cross-border treatment (treatment access via the European Social Security Regulation 883/04 and 987/06 – S2 route) and set-up and maintenance of the treatment center.

Conclusion:
The timing for implementing C&G therapy is unpredictable, and the window for treatment is often short, requiring rapid action from a newly established team. Especially cross-border successful treatment requires specific skills, project coordination, and stakeholder approval within a European environment. Additionally, there are many processes involved, requiring constant updates among various stakeholders, from patients to payers. It's possible to streamline the process and ensure timely treatment by building a network of experts, presenting diagnostic and eligibility criteria, and leveraging external teams, when necessary. Hospitals should allocate internal resources (in-house C&G therapy team) or consider external companies to handle processes.

P706

Gene Editing induced Gene Silencing (GEiGS) - A new platform for programmable cell engineering

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Cell therapies are showing considerable clinical promise but face significant challenges relating to efficacy, safety, and cost. Clearing these hurdles requires increasingly complex cellular engineering. We have established a new gene silencing approach that uses minimal genome edits (~50 base pairs) to elicit tunable gene knock-down that is programmed to be dependent on a specific cell state (i.e linked to identity or environment): Gene Editing induced Gene Silencing (GEiGS). Here we show that GEiGS is an effective technology able to direct stable, tunable and programmable gene expression changes for development of allogeneic and autologous cell therapies.

GEiGS is a new way of leveraging the cell's RNAi pathway to silence gene expression. Our computational platform recodes endogenous miRNAs towards a new desired gene target. Notably, arrays of designs are computed that result in various degrees of silencing. This is achieved through a combination of trigger (anti-sense) design and miRNA scaffold selection and engineering. The endogenous miRNA that is edited is chosen based on its expression profile, enabling activity of our engineered miRNA only in a particular cell state. Furthermore, physiological expression levels, miRNA network redundancy and minimal gene editing result in no detectable loss or gain of function effects.

We present data on GEiGS implementation in iPSC, iPSC-derived pancreatic progenitors and macrophages, and in primary T-cells, demonstrating its potential in autologous and allogeneic settings. Targets include components of the MHC-I complex and genes involved in immune cell function within the solid tumour microenvironment, enabling development of CAR-T or macrophage based therapeutics with improved efficacy and safety profiles.

P707

Identification of Novel γ-globin Repressors Through a Custom CRISPR Knockout Screen and Validation Studies for the Treatment of β-hemoglobinopathies

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Haemoglobinopathies are a group of conditions affecting haemoglobin. The haemoglobin tetramer consists of two α- and two β-globin chains ($\alpha_2\beta_2$). Mutations in the *HBB* gene, which encodes for the β-globin chain, cause two of the most common inherited monogenic disorders, β-thalassaemia and sickle cell disease. These diseases affect millions of individuals worldwide, with Cyprus having the third highest percentage (12%) of β-thalassaemia carriers. Reactivation of the γ-globin gene for the production of fetal haemoglobin (HbF) is a promising therapeutic strategy. Gene therapy approaches related to the elevation of HbF levels are in clinical trials, however, associated risks, costs and availability limit their use. Additionally, pharmacological targeting of *BCL11A* and *LRF* (*ZBTB7A*), which are the main transcription factors regulating γ-globin levels, is difficult, and is complicated by the fact that these genes are also involved in the regulation of multiple non-erythroid genes. Thus, identification of new factors amenable to pharmacological control for the treatment of β-haemoglobinopathies is of utmost importance.

Several candidate genes were identified based on a custom CRISPR/Cas9 knockout screen, which targeted 293 genes selected from previously published literature. The screen identified seven potential γ-globin repressor genes, which scored as highly during the screening process as some of the well-known γ-globin regulators. The three most promising candidate genes have been selected for further validation and functional studies. *Gene A* encodes for a protein involved in ion transport and iron homeostasis, *Gene B* is a transcriptional regulator and *Gene C* plays a central role in chromatin remodelling and acts as a transcriptional regulator.

These genes are being validated individually through CRISPR/Cas-mediated knockouts based on lentiviral transduction as well as nucleofection with ribonucleoproteins in the HUDEP-2 cell line. After expansion, the cultures are subjected to erythroid differentiation for 9 days. The editing efficiency is assessed at the DNA level using the Interference of CRISPR Edits (ICE) web-tool at several timepoints throughout erythroid differentiation. In addition, immunoblotting is utilised for detection and quantification of the candidate genes, as well as the α- and γ-globin chains on different days of the erythroid differentiation.

The individual CRISPR/Cas-mediated knockouts had high editing efficiencies (80%-87%) for all three of the candidate genes. However, based on the immunoblots, the individual knockouts were unable to abolish protein expression of *Genes A* and *B*. In the literature, exon skipping has been reported as one of the main mechanisms causing inconsistency between high editing efficiency at the DNA level and failure of protein expression knockout. Currently, cDNA sequencing of *Gene A* and *Gene B* is carried out in order to investigate for exon skipping. In parallel, a two-gRNA duplex strategy is utilised, where two gRNAs which target two different exons are used for nucleofecting HUDEP-2 cells, aiming to cause a large deletion.

In conclusion, seven new candidate γ-globin repressor genes have been identified through the use of a CRISPR/Cas9 knockout screen. Validation studies are ongoing for three genes and studies of their mode of action and usefulness as therapeutic targets will follow.

P708

Simultaneous inhibition of DNA-PK and PolΘ improves integration efficiency and precision of genome editing

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Genome editing, particularly CRISPR/Cas9 technology, have transformed biomedical research and opened opportunities for developing curative treatments for genetic diseases. Despite rapid progress, the low efficiency of targeted DNA integration and the generation of undesired mutations represent major limitations for genome editing applications caused by the interplay between the main DNA Double-Strand Break (DSB) repair pathways: Homology-Directed Repair (HDR), Non-Homologous End Joining (NHEJ), and alternative-End Joining (alt-EJ). To address this, we intended to identify new targets to improve the efficiency of targeted genome insertions and conducted a large-scale compound library screen for various potential contributors. We identified DNA-dependent Protein Kinase (DNA-PK) as the most effective target to improve CRISPR/Cas9-mediated genome insertions, confirming previous findings. This led to the detailed characterization of AZD7648, a selective DNA-PK inhibitor, as a potent enhancer of CRISPR/Cas9-mediated integration. We demonstrated that AZD7648 increased HDR and decreased mutagenic NHEJ repair, thus resulting in improved performance of precise gene editing. Furthermore, we observed additional improvement of integration efficiency by impairing Theta-mediated End Joining (TMEJ), a form of alt-EJ repair, through DNA polymerase Θ (PolΘ) inhibition. Combined treatment with AZD7648 and PolΘ inhibitors (which we named 2iHDR) substantially increased the precision of templated insertions, with efficiencies of up to 80% and nearly no formation of undesired Insertion-Deletions (InDels). Notably, 2iHDR also reduced Cas9-associated off-target activity, greatly improving the performance and fidelity of CRISPR-Cas9 gene editing.

P709

Base editing mediated splice site disruption of *FKBP12* to induce tacrolimus resistance in T cells

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Transplant recipients are at risk of severe morbidity through infections because of the life-long immunosuppression. In particular herpes viruses can cause life-threating diseases, including CMV pneumonia and EBV-associated post-transplant lymphoproliferative disease. Virus-specific T cell therapy can be used for prophylaxis and treatment of such infections, but the immunosuppressive drugs will also limit the therapeutic efficacy of any adoptively transferred T cell. To overcome this

challenge, we recently demonstrated that CRISPR-Cas9-mediated knock-out of FK506-binding protein 12 (*FKBP12*) effectively induces the resistance to the calcineurin inhibitor Tacrolimus in virus-specific T cells without compromising their functionality in vitro. Therefore, gene editing is a promising strategy to improve the functionality of T cells in patients receiving Tacrolimus-based immunosuppression. However, conventional gene editing techniques rely on inducing DNA double strand breaks (DSB), which may lead to unintended on-target mutations and possible larger genetic rearrangements. Base editing is a gene editing method that directly generates precise point mutations in genomic DNA without generating DSB, which is more precise and minimizes the formation of DSB-repair associated byproducts.

Here, we tested whether DSB-free CRISPR-Cas9 adenine base editing can be used to knock-out (KO) *FKBP12* in T cells. We screened and identified single guide RNA (sgRNAs) that may decrease FKBP12 protein expression by disrupting splice acceptor or splice donor sites. To design sgRNA for base editing, we employed SpliceR (http://z.umn.edu/spliceR) searching for suitable sgRNA within the *FKBP12* gene. Chemically-modified gRNAs and base editor mRNA were co-electroporated into activated primary human T cells. Sanger sequencing and editR analysis (baseEditR.com) was performed to compare the editing efficiency of the base editing on a DNA level. Multiple sgRNA were tested with adenine base editors targeting various PAMs (NGG, NG). We identified one sgRNA which enabled 100% efficient editing at the splice site on DNA level, when targeted with an 8th generation adenine base editor (ABE8.20m) using a conventional Cas9 nickase (NGG PAM). Next, we will investigate the functional impact of the base edits in comparison to *FKBP12*-KO T cells generated by conventional CRISPR-Cas9. Additionally, we will explore the optimization of transfection using lipid nanoparticles (LNPs) to avoid electroporation dependent toxicity during base editing. Optimization of base-editing mediated *FKBP12*-KO may improve the safety of future T cell products by avoiding translocations and other genetic rearrangements which arise from DSB. In the future, these results may enable efficient multiplex editing of T cells to combine Tacrolimus resistance with other desirable features for improved cellular activity in transplant patients.

P710

Modelling the generation of oncogene-carrying extrachromosomal circular DNA through CRISPR-C

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State of the art sequencing methods opened broad perspectives for exploring the landscape and varied structure of extrachromosomal circular DNA (eccDNA), a prominent form of focal gene amplification frequently associated with the development of cancer and malignancy. However, the vast amount of data and emerging theories resulting from these sequencing efforts have underscored the urgent need for a reliable approach to model the generation and amplification of recurrent oncogene-carrying eccDNAs. In our laboratory, we have devised a molecular tool called CRISPR-C, which enables precise generation of eccDNA by deleting specific chromosomal regions. To enhance the efficacy of eccDNA generation, we have adapted CRISPR-C by directly

delivering the Cas9 enzyme and two editing sgRNAs as a ribonucleoprotein (RNP) complex. Leveraging our eccDNA-biosensor cell model, which allows us to scrutinize the biogenesis of a particular fluorophore-encoding eccDNA in human cells, we have observed a substantial 6-7 fold increase in eccDNA generation efficiency. Employing the modified CRISPR-C, we have successfully generated eccDNAs harbouring oncogenes (e.g., EGFR, CDK4, MDM2, or DHFR) across a spectrum of cell types, including HEK-293T, U2OS osteosarcoma, MCF7 breast cancer cells, primary human astrocytes, and skin fibroblasts. By employing DNA Fluorescent In Situ Hybridization, we visualized deletion of the targeted chromosomal region and have ascertained the extracellular presence or chromosomal relocation of the eccDNA. Furthermore, we have demonstrated that our system is capable of generation of chimeric eccDNAs composed of target sequences from distant intra or inter-chromosomal regions. Upon induction of CDK4 and MDM2-carrying eccDNA formation, we have observed diminished viability and proliferation capacity of U2OS cells one-week post-procedure, with recovery apparent after three weeks. This effect has corresponded with a global downregulation in transcriptional activity of genes located within the CRISPR-C target regions across most experimental groups, subsequently restored after an additional two weeks, thus indicating prevalent gene deletions at that specific time point. By integrating the refined version of CRISPR-C with our eccDNA biosensor system, we have elucidated the impact of suppressing various DNA repair and DNA sensing-related genes on eccDNA generation. Our findings highlight the significant but non-exclusive role of DNA-PKCs, the principal kinase involved in non-homologous end-joining, in eccDNA biogenesis. Additionally, we have discovered the involvement of DNA ligase 4 in the reintegration of the generated DNA fragment, while intriguingly not in the circularization of the eccDNA.

P712

Gene editing approaches as potential treatments for Alexander disease

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Alexander disease (AxD) is a rare, lethal leukodystrophy caused by dominant mutations in the gene encoding for glial fibrillary acidic protein (GFAP), the main intermediate filament of astrocytes. Accumulation of GFAP aggregates in Rosenthal fibers leads to Central Nervous System (CNS) dysfunction with typical pathological traits such as astrogliosis, loss of myelin, seizures, spasticity, and megalencephaly. No cure is currently available for this neurodegenerative disorder.

Here, we aim at developing novel, single-dose gene editing strategies for the lifetime treatment of AxD. To this end, we selected a single guide RNA (sgRNA) targeting the murine *Gfap* gene in murine 3T3 cells transduced with a lentiviral vector (LV) harboring the R76H-mutant GFAP protein fused to mCherry. FACS analysis of mCherry expression showed that the best sgRNA candidate induced a robust knock-down of GFAP-mCherry, 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, pilot experiments defined the optimal AAV serotype and promoter, resulting in high astrocytic tropism and transduction rates of AxD-affected brain regions. Then, AAV carrying the *Gfap*-targeting sgRNA and Cas9 nuclease were administered by intracerebroventricular injections in neonatal AxD mice. AVV-mediated Cas9/sgRNA delivery resulted in on-target editing in GFAP$^+$ astrocytes,

which was confirmed at a protein level by a decrease in GFAP expression at 3 months post-treatment. Importantly, Cas9/sgRNA-treated animals showed a mitigated accumulation of Rosenthal fibers - a hallmark of AxD pathology - in white matter regions. These data provide *in vivo* proof-of-concept of the efficacy of a CRISPR/Cas9 editing approach in ameliorating disease-associated phenotypes.

To expand on the potential of gene editing as a treatment for AxD and avoid aberrations associated with permanent GFAP knock-out (e.g. altered synaptic transmission and reduced myelination), we are currently generating an allele-specific gene therapy targeting the murine R76H and R236H mutations, homologs of the human mutation hotspots detected in AxD patients. We identified Cas9/sgRNA that induce allele-specific knock-out of mutated *Gfap* sequences. We additionally identified adenine base editors that correct the R76H mutation, thus reducing the risk of genotoxicity associated with Cas9 nucleases.

Overall, these results pave the way for advanced preclinical studies aimed at the safe and effective delivery of editing systems targeting the mutated *Gfap* allele in the CNS using AAV vectors or, prospectively, nanoparticles. 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.

P713

Development of a CRISPR-Cas9 system for *ATXN3* gene editing in Spinocerebellar ataxia type 3 disease

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Spinocerebellar ataxia type 3 (SCA3) is a rare neurodegenerative disease caused by an unstable CAG repeat expansion within the exon 10 of *ATXN3* gene. The accumulation of mutant ataxin-3 protein in neurons leads to prominent cell death in the cerebellum and other brain regions. SCA3 patients present with cognitive and motor symptoms, which worsen with disease progression. Being the monogenic disease with only symptomatic treatment available, *ATXN3* represents an attractive therapeutic target for gene editing. Here, we implemented CRISPR/Cas9 system for *ATXN3* editing as a potential SCA3 therapy. We explored a non allele-specific approach, such that all SCA3 patients would be eligible. We aimed to eliminate both wild-type and mutant *ATXN3* gene by targeting its translational start site in exon 1. For this, we employed AAV-mediated co-delivery of CRISPR/Cas9 and one sgRNA in the deep cerebellar nuclei of MJD84.2 mice. We showed high *ATXN3* editing efficiency *in vivo*, reaching up to 50 %. Additionally, the “Ablate and Replace” strategy was assessed, delivering a CRISPR-resistant wild-type *ATNX3* cDNA, to prevent potential detrimental effects of gene ablation. For an improved safety profile, self-inactivating system was constructed, allowing transient expression of Cas9 in the brain. This was achieved with another sgRNA targeting the Cas9 sequence. Together, this study demonstrates the potency of CRIPSR/Cas9 for *ATXN3* editing in the mouse cerebellum, underpinning a promising therapeutic option for future clinical applications.

P714

CRISPR activation as a therapeutic tool for Spinocerebellar ataxia type 3 (SCA3)

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Spinocerebellar ataxia type 3 (SCA3) is a neurodegenerative disorder caused by an expansion in the CAG repeats in the *ATXN3/MJD1* gene, resulting in an expanded polyglutamine tract in the ataxin-3 protein. Ultimately, this results in neurodegeneration in different brain regions, such as the cerebellum, the brainstem, and the striatum. Neuropathological features progressively evolve to incapacitating symptoms.

Previously, our group demonstrated the relevance of caloric restriction in blocking the SCA3-associated neuropathology. These effects were mainly mediated by sirtuin 1 and, in fact, both mouse models of SCA3 and fibroblasts of patients showed decreased levels of sirtuin 1, that were re-established by caloric restriction. Moreover, overexpression of sirtuin 1 using lentiviral vectors reduced ataxin-3 aggregation and blocked neurodegeneration in a SCA3 mouse model. Therefore, increasing sirtuin 1 levels in the context of SCA3 is a potential therapeutic strategy that should be explored by gene therapy approaches, such as CRISPR activation (CRISPRa).

CRISPR-Cas9 specificity and safety has already been proven. More than gene editing, CRISPR technology was repurposed to efficiently regulate gene expression in preclinical studies, offering the possibility to regulate several genes simultaneously. Since it does not perform double stranded breaks in DNA, CRISPRa is a safe approach which lacks everlasting DNA alterations. In the present work, we aimed at increasing sirtuin 1 expression in the context of SCA3, by developing a CRISPRa system targeting the sirtuin 1 promoter.

To achieve that, sgRNAs targeting the upstream region of the transcription starting site were designed using an *in silico* platform. Screening of sgRNAs was implemented in HEK293T by transfection with CRISPRa plasmids: 1) one containing Tdtomato under the control of either mouse or human sirtuin 1 promoter; 2) another encoding “dead” Cas9 from *staphylococcus aureus* fused to VP64, P65 and Rta (sadCas9-VPR) and 3) a plasmid expressing blue fluorescent protein (BFP) as a control of transfection efficiency.

After analysis, the sgRNA showing higher activation of mouse sirtuin 1 promoter led to a 3-fold increase in Tdtomato fluorescence. The reporter levels were even higher upon transfection with two sgRNAs simultaneously, including the combination of sgRNAs #1 plus #5 and sgRNAs #1 plus #4. In these cases, Tdtomato expression increased 5 times when compared to a scramble sequence. In the case of human sirtuin 1 promoter, the sgRNAs #2, #4 and #9 showed the highest levels of Tdtomato, and the combination of two sgRNAs achieved around 3-fold increase.

This allowed us to select the top sgRNAs that were then validated *in vitro* regarding the efficiency of endogenous activation. Mouse sgRNAs were validated in Neuro2a cells and human sequences in HEK293T cells. Both experiments showed the highest expression of sirtuin 1 when using two sgRNAs simultaneously in accordance with the screening assay. In fact, mouse sirtuin 1 mRNA levels increased up to 20 times and protein levels increased 3 times, while human sirtuin 1 mRNA levels showed up to 2.5-fold increase when compared to a scramble sequence. The present results

support the potential of targeting sirtuin 1 using CRISPRa in SCA3 and possibly other polyglutamine disorders.

P715

CRISPR/Cas9-based genome editing for correction of X-linked Emery-Dreifuss Muscular Dystrophy

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Type I EDMD is a rare genetic X-linked disease caused by mutations in the *EMD* gene, encoding emerin. A cure for this disease is not available to date and the molecular pathogenesis of EDMD1 is not entirely elucidated. The identification of biomarkers for the evaluation of disease progression is mandatory.

Herein, we corrected two genetic mutations in the *EMD* gene by CRISPR/Cas9 technology. CBE was used to correct a non-sense mutation in the N-terminal domain. To evaluate the genomic correction, NGS analysis was performed and showed high frequency of base editing-mediated repair in patient fibroblasts and myoblasts. The corrected EDMD1 cells showed the restoration of emerin expression and the protein was properly localized in the nuclear membrane. As the LINC complex is considered a main driver of nuclear envelope-related mechanisms in developing skeletal muscle, we investigated SUN1 localization in EDMD1 myotubes. SUN1, farnesylated prelamin A and pericentrin were mislocalized and after CBE a complete rescue was observed.

We also corrected a mutation affecting the C-terminal domain of emerin leading to a frameshift of *EMD* gene and generating a truncated protein missing the transmembrane domain. We designed a CRISPR-based strategy to re-establish the correct frame of the gene in patient's tenocytes, confirmed by NGS analysis on treated cells. Moreover, after CRISPR treatment, emerin localization in the nuclear envelope was obtained in approximately 15% of treated cells. In conclusion, we corrected genetic defects in EDMD1 cells that could represent isogenic controls used to identify biomarkers for the evaluation of disease progression.

P716

He-RASE: a fast cellular model to screen engineered CRISPR/Cas systems editing dominant mutations

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The CRISPR/Cas system holds great potential for the treatment of many genetic disorders. In particular, it can tackle autosomal dominant mutations through the disruption of mutant alleles via the Non-Homologous End Joining (NHEJ) DNA repair pathway. However, due to the low specificity of the Cas nuclease, many single-nucleotide dominant mutations cannot be targeted by

CRISPR/Cas because of the lack of discrimination between mutated and wild-type alleles. Therefore, to expand the range of dominant diseases that can be addressed by the CRISPR/Cas system, it is crucial to develop a highly specific Cas nuclease capable of an efficient allele-specific single-nucleotide discrimination.

Here, we propose to use structure-guided mutagenesis approach to engineer CRISPR/Cas nucleases to tackle specific TP63 heterozygous missense mutations causative of Ectrodactyly-Ectodermal Dysplasia-Cleft lip palate (EEC) syndrome. EEC is characterized by a triad of ectrodactyly, ectodermal dysplasia and facial clefting together with a wide spectrum of other clinical manifestations (e.g. corneal blindness). Surgery can improve some aspects of patients' lives, but the main unmet medical needs remain severe visual impairment and blindness, caused by corneal opacification due to progressive limbal stem cell deficiency.

To functionally screen the engineered CRISPR/Cas, we generated He-RASE (**He**k293T **R**eporter-based **A**llele-**S**pecific **E**diting) screening tool, a HEK293T-based cell line that recapitulates the "biallelic context" of the dominant disease. Our cellular model accurately mimics the biallelic context of EEC patients' TP63 loci enabling us to effectively screen the engineered CRISPR/Cas nucleases. This screening will allow us to identify the most promising candidates both for the editing efficiency and the allele-specificity on the mutations of interest.

The identification of efficient and specific nucleases would widen the range of dominant genetic diseases potentially targeted by CRISPR/Cas. The validation of our proposed cellular model will serve as a first step to confirm the feasibility of the gene editing approach in patients' primary cells and to set up an *ex-vivo* gene therapy for the corneal damage of EEC patients.

P717

INDUCE-seq: Ensuring the safe development of cell and gene therapies by gene editing

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Advances in genome editing are making it increasingly possible to develop new cell and gene therapies. Synthetic genes and modifications to existing ones can be made in a site-specific manner, while immune-compatible cells created for use in allogeneic patients enable production of 'off-the-shelf' cellular therapies. However, gene editing is a new way of treating disease, requiring new tools to ensure their safe and efficacious use in patients. Genome editing comes with risk, including malignant transformation of target cells caused by culturing cells outside the body. This can introduce mutations that confer growth advantages to cells. Furthermore, gene editing can directly cause genomic instability. In cells harboring pre-existing DNA repair defects, the selection for outgrowth of clones with oncogenic mutations may result. Finally, the gene editing tools themselves can cause DNA breaks in the genome at sites other than the intended target. Such off-target editing could activate proto-oncogenes, or disrupt tumor suppressors thus driving carcinogenesis. Off-target mutagenesis may also generate neoantigens, triggering autoimmunity, or other types of cellular dysfunction. Furthermore, rare penetrant mutations are now known to confer severe risk of common disease, underscoring the importance of identifying off-target gene editing. More precise methods are needed for testing off-target editing during all phases of therapeutic development, including treatment follow-up. At present standardised assays to assess the safety of gene editing-based therapies are lacking. Here, we describe the development and characterization of INDUCE-seq to address this. CRISPR-Cas9-based gene

editing of five well-studied genetic targets was conducted by two independent industry partners, using two different cells types. On and off-target gene editing was assessed by measuring breaks in the genome using INDUCE-seq. The genetic changes at these locations were subsequently measured using Duplex Sequencing, which allows for sensitive detection of exceptionally rare mutations. This work was conducted in collaboration with members of the HESI CT-TRACS consortium. The results of the study will be presented.

P718

Culture-free gene editing of hematopoietic stem and progenitor cells for the gene therapy of β-hemoglobinopathies

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CRISPR-Cas9 gene editing of patient hematopoietic stem and progenitor cells (HSPCs) effectively induces fetal hemoglobin (HbF), ameliorating β-hemoglobinopathies. Among the HbF-inducing gene editing targets under clinical investigation, we found that disruption of the 11kb *PRR-βE1* region in the β-globin cluster alters chromatin looping and results in robust HbF activation. *Ex vivo* manipulation of the autologous HSPCs involves the pre-stimulation with cytokines for up to 72 hrs. The loss of HSCs quiescence on *ex-vivo* culture results in proliferation, decreasing the engraftable gene-modified cells *in vivo*. Unlike lenti viral strategies, gene editing strategies exploiting non-homologous end joining may not require HSPCs pre-stimulation. In this study, we have examined whether *PRR-βE1* gene editing is feasible without cytokine pre-stimulation. On comparing the uncultured day 0 HSPCs to the standard protocol involving 48hrs culture, we showed comparable *PRR-βE1* editing and HbF activation. Functionally, both *PRR-βE1* edited un-cultured and 48hrs cultured HSPCs showed comparable engraftment, multilineage differentiation potential, and HbF^{+ve} cells *in vivo. These results indicate that PRR-βE1 gene editing is feasible without ex vivo culturing of HSPCs.*

P719

CRISPRsim: A toolkit for creating simulated genome-edited samples in sequencing data

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DNA sequencing of genome-edited cell populations is useful in detection and characterization of editing events. Sequenced reads are then analyzed with computational tools such as mutation callers to quantify editing events. Many mutation callers are well-validated in non-genome-editing fields such as the detection of germline or somatic mutations, but their accuracy or efficacy in detecting genome editing outcomes not been extensively studied. Here, we present CRISPRsim, a

toolkit for creating simulated genome-edited samples, which we believe will aid in the validation of existing computational tools and the development of novel tools that are specific to the genome editing space.

CRISPRsim introduces mutations into an unedited sample. The unedited sample is in the form of aligned sequencing data and may be from amplicon sequencing, whole-genome sequencing, or long-read sequencing. Edits can be specified manually (e.g. to create a sample with a specific edit) or amplicon sequencing data from a genome-edited sample can be provided and the edits from that sample are introduced into the reads of the unedited sample. Users can specify the rate at which edits are introduced into the simulated sample as well as the sequencing read depth of the simulated sample.

We used CRISPRsim to generate simulated samples of varying edit frequencies and sequencing depths, which we used to test four commonly-used mutation callers. Some tools exhibited high sensitivity in identifying simulated editing events, but they also incorrectly reported sequencing errors and other noise that originated in the unedited sample. Conversely, other tools did not report these sequencing errors but were too stringent and filtered out genuine genome-editing events for example due to high allele diversity at that location. We utilized the

In summary, CRISPRsim is a useful tool for creating simulated samples that contain edits observed in genome-edited samples. Simulated samples can be used to evaluate the utility, sensitivity, and specificity of existing mutation callers in identifying genome editing. Additionally, simulated samples may be useful in developing novel genome-editing-specific computational tools which will improve the field's ability to detect and characterize genome editing events.

P721

Therapeutic genome editing of Cystic Fibrosis using Prime Editing and PRINS

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Cystic fibrosis (CF) is a genetic disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. It affects the lungs, pancreas, and other organs in the body. Current treatments for CF (e.g. Trikafta and gene therapy approaches) target only a specific subset of patients and additional therapies or redosing are needed. Genome editing with CRISPR-Cas9 has been used to correct the CFTR gene, but its efficiency in vivo is low. Prime editing (PE) uses an enzyme called a prime editor to directly insert or delete specific nucleotides within the DNA, without making a double-stranded break (DSB). This allows for more precise and efficient editing and reduces the likelihood of unintended mutations.

AstraZeneca has developed two strategies for introducing precise genomic insertions using the SpCas9 nuclease-based prime editor, PEn, but more work is needed to optimize the system for therapeutic use. The first approach combines PEn with canonical pegRNAs to promote a homology-dependent DSB repair leading to precise insertions. The second approach, called PRINS, uses springRNAs that do not require a homology sequence in the reverse transcriptase (RT) template and the intended insertion is installed through a precise non-homologous end-joining pathway (NHEJ) (Peterka et al., 2022). Here, we plan to install two CF- mutations in human cell lines that are expressing CFTR (e.g. DLD1 cell line), and afterward optimize PEn and

PRINS approaches to develop a therapeutic genome editing treatment for CF in human cell lines and patient-derived intestinal organoids. Moreover, we plan to use AstraZeneca proprietary lung targeting lipid nanoparticles (LNP) to test PEn in vivo in mice and perform safety and toxicity tests.

P722

Highly efficient bi-allelic correction of homozygous COL7A1 mutation with base editing in Recessive Dystrophic Epidermolysis Bullosa patient fibroblasts

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Base and prime editors are novel CRISPR variants that utilise single-stranded DNA cleavage, elegantly bypassing the need for homology-directed repair and the risk of indels by non-homologous end joining. This reduces the risk of significant indels at on target and off target loci. As a result, these tools offer great therapeutic potential for inherited skin diseases such as recessive dystrophic epidermolysis bullosa (RDEB). Bi-allelic loss-of-function mutations in *COL7A1* cause RDEB, resulting from loss of functional type VII collagen (C7) in the basement membrane zone. Current therapies for RDEB are limited, but *ex vivo* and *in vivo COL7A1* base or prime editing therapies can potentially restore normal protein production with a single intervention treatment. In this study, we aim to compare the advantages and disadvantages of base and prime editing as tools for correcting *COL7A1* c.4448 G>A (p.Gly1483Asp) homozygous mutations in RDEB patient fibroblasts. In base editing, we have demonstrated up to ~90% efficiency of correction with no bystander effects using ABE8e mRNA with Lipofectamine Messengermax. First, adenine base editor (ABE8e) mRNA together with single guide RNA (sgRNA) were transfected into patient primary fibroblasts with Lipofectamine MessengerMax. By varying concentrations and ratios of guide RNA and ABE8e mRNA, we have achieved ~90% bi-allelic correction without introducing any bystander mutations using 400ng of ABE8e and 57ng of sgRNA by transfecting 100,000 cells. In our prime editing work, we have established a proof-of-concept protocol and achieved up to ~60% editing efficiency with PEMax plasmid and a published guide in HEK293T cells. Our next steps are prime editing to correct RDEB causing mutations in patient fibroblasts and compare the editing efficacy with ABE8e. Initial prime editing experiments are underway with PEmax mRNA and engineered prime editing guide RNAs (epegRNA) to optimise the guide design and to compare PE2max, PE3max, PE4max and PE5max editing systems. Further, we will investigate the off target effects of base edited and prime edited RDEB primary fibroblasts with RNA-Seq and top 10 off target DNA sequencing and characterise edited cells in terms of protein production and secretion, adhesion and proliferation. Taken together, so far we have established a pipeline which demonstrates potential value for base editing as gene editing tools in RDEB as gene therapeutics and we aim to do the same for prime editing now.

P723

Adenine-Base-Editor-mediated correction of KRAS and TP53 gene mutations in pancreatic cancer

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Cancer is among the leading causes of death. Despite the established classical modern treatment modalities, including surgery, chemotherapy and irradiation, pancreatic cancer has a dismal 3-year survival rate of less than 10%. Gene therapy presents a promising avenue especially as two of the most common cancer-causing gene mutations comprise the *TP53* and *KRAS* missense mutations, both of which are notorious to target using conventional therapies. The invention of CRISPR-Base-Editing made single-base conversions without generating DNA double strand breaks possible. Our group has recently demonstrated the depletion of mutated cancer cell lines and patient-derived organoids upon CRISPR-mediated correction of *KRAS* and *TP53* mutations utilizing a lentiviral approach to transduce the Adenine Base Editor (ABE) and the gRNAs. Here we aim to position the system towards clinical translation and test whether it would be possible to deliver the ABE and gRNA complex using a lipid-mRNA-based delivery approach. For this purpose, cells of the cancer cell line PANC-1 harboring point mutations in $KRAS^{G12D}$ and $TP53^{R273H}$ were infected with a lentivirus containing the ABE8e, GFP and Puromycin-resistance gene. After selection in Puromycin, cells were transfected using the transfection reagent “Invivo-JetRNA” (Polyplus, France) using each 100 pmol gRNA targeting the $KRAS^{G12D}$ and $TP53^{R273H}$ mutations. Over a period of up to 2 weeks, cells were cultured to monitor the depletion following successful mutation repair. For this purpose, gDNA was isolated at different timepoints to determine the editing efficiency and the percentage of edited cells. The surviving cells were identified by Crystal Violet staining to visualize the contrast in viability between mutation-targeting gRNA conditions and the non-targeting control gRNA. We also included the RKO cell line, which was infected with the same lentivirus, but does not harbor the two mutations. Transfection rates with “Invivo-JetRNA” exceeded 90% and editing efficiency for $TP53^{R273H}$ after one single transfection reached up to 71%. Interestingly, the editing efficiency for $Kras^{G12D}$ was lower with around 30%, even though the same amount of gRNA concentration was used. After almost two weeks only around 15% of $TP53^{R273H}$ edited cells were still alive. Interestingly, using a completely transient approach by transfecting ABE8e-mRNA and the mutation-targeting gRNAs simultaneously using “Invivo-JetRNA”, a considerable level of editing was also observed. Taken together, these results demonstrate a promising potential for cancer gene therapy that warrant in in-vivo applications in a rodent model as the next step.

P724

Extinction of all infectious HIV in cell culture by the CRISPR-Cas12a and b systems

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The CRISPR-Cas9 system has been utilised successfully for genome editing of various organisms. We previously reported the inhibition of HIV in cell culture infections and subsequent viral escape when a single guide RNA (gRNA) was employed, but complete inactivation of all infectious HIV

was achieved with specific combinations of two gRNAs. Although this outcome is remarkable, the large size of CRISPR-spCas9 transgene cassettes hampers their implementation in gene therapy applications with vectors that possess limited packaging capacity, including lentiviral vectors (LV). Therefore, there exists a pressing need for simpler and smaller CRISPR-Cas vector designs, for which the Cas12a and Cas12b nucleases are attractive candidates. CRISPR-Cas12 presents several distinctive features. For example, Cas12 targets a T-rich PAM sequence, thus expanding the potential target sequences when compared to Cas9. Cas12 produces a sticky DNA end that holds potential for HIV inactivation, as the cleaved and subsequently repaired DNA sequence is likely to be re-cleaved by Cas12 due to the retention of critical recognition motifs. Such subsequent rounds of DNA cleavage and repair will result in more pronounced mutations at the cleavage site, thereby enhancing the likelihood of HIV inactivation.

We compared Cas12a and Cas12b with the original Cas9 system for the purpose of inactivating the integrated HIV DNA genome. Anti-HIV gRNA molecules, which are fully complementary to the highly conserved region of the primary virus isolate LAI, were designed using the Benchling CRISPR Guide Design Software. Initially, we assessed the anti-HIV effect in transient assays, where HIV LAI DNA (300 ng) was co-transfected into HEK293T cells with plasmids encoding the Cas12a/Cas12b endonuclease and a single gRNA. Subsequently, we stably transduced SupT1 cells with lentiviral vectors that encode both CRISPR components. For each gRNA-expressing cell line, we infected six parallel cultures with HIV LAI, and the emergence of virus-induced syncytia was monitored for a period of up to 60 days. Representative cultures were selected for a genetic analysis of the HIV genome, in which we expected to identify lesions caused by CRISPR/Cas-editing that could elucidate the curative phenotype. Notably, Cas12a exhibited superior antiviral activity and achieved complete HIV inactivation in cell culture with only a single gRNA (referred to as crRNA). We disclose that DNA cleavage by the Cas12 endonuclease and subsequent DNA repair leads to mutations with a sequence profile that is distinct from that of Cas9. While both CRISPR systems can induce the typical small deletions, Cas12 does not induce the pure DNA insertions that are commonly observed with Cas9.

In conclusion, we have demonstrated that Cas12a and Cas12b can accomplish full HIV inactivation in cell culture using only a single gRNA through "hypermutation" at the target site, facilitated by DNA cleavage and subsequent error-prone DNA repair. We propose that the differential outcome can be attributed to the distinct architecture and kinetics of the Cas9 and Cas12a nucleases.

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P725

Prime editing of human CD34+ hematopoietic stem cells for correction of GATA2 deficiency and X-linked chronic granulomatous disease

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Ex vivo gene editing of autologous human hematopoietic stem cells is currently being developed as a treatment for inborn errors of immunity (IEI), but the current gene editing platform relying on Cas9 and the use of rAAV6 as a donor for homology-directed repair is challenged by indel formation, off-target editing and toxicity in hematopoietic stem cells. Thus, there is a need for versatile genome editing strategies in hematopoietic stem cells that do not require delivery of a donor template and works without generating DNA double-strand breaks (DSB). Here, we present ongoing work using the DSB-independent prime editing technology to correct several patient-derived IEI-causing mutations. We optimized prime editing guide RNAs (pegRNAs) to efficiently correct mutations in a panel of disease genes, including *GATA2* and *CYBB*, causing GATA2 deficiency and X-linked chronic granulomatous disease, respectively. We also optimized nicking sgRNAs (ngRNAs) for both *GATA2* and *CYBB* and found that editing efficiencies were highly dependent on the ngRNA, with prime editing rates in model K562 cell lines varying between 20% and 80% with alternative ngRNAs. In vitro transcribed PEmax mRNA as well as synthetic pegRNAs and ngRNAs were co-delivered to patient-derived peripheral blood mononuclear cells as well as CD34+ hematopoietic stem and progenitor cells (HSPCs) from healthy donors, yielding prime editing rates of 20% and 35%, respectively. Notably, while prime editing generally resulted in less editing than conventional HDR-based gene editing, prime edited HSPCs showed superior viability and significantly increased colony-forming potential. This indicates an increased fitness compared to traditional CRISPR-based gene editing approaches, supporting the further exploration of prime editing to treat IEI.

P726

GUMM: A purpose-built computational workflow for single cell genotyping of gene editing experiments

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CRISPR-Cas9-based gene editing is a powerful approach to improve our ability to treat specific diseases with an unmet medical need. Engineering cell therapies requires accurate assessment of gene modified allelism because editing patterns can vary across cells and cause phenotypic heterogeneity in the final drug product. This can delay development of complex cell therapies involving the use of multigenic editing. Recently, droplet-based targeted single cell DNA sequencing (scDNAseq) has been used to genotype select loci across thousands of cells enabling high-throughput assessment of gene editing efficiency. Here, we developed a novel computational workflow called GUMM (Genotyping Using Mixture Models) that systematically infers single cell allelism from scDNAseq data by fitting a series of Gaussian mixture models to allele read counts

generated by CRISPResso2. It is uniquely well-suited for analyzing gene editing experiments where cells in the sample are genetically homogenous and differ only at the editing site(s). GUMM outputs a probabilistic prediction of cell editing genotype and is aware of technical artifacts including low coverage at the editing sites, PCR amplification imbalance, doublets, and sequencing errors. This obviates the need to set arbitrary edited allele frequency thresholds to determine cell heterozygosity. Furthermore, GUMM can accurately identify doublets using only information from edit sites to flag cells based on ploidy and likelihood of allele co-occurrence. To assess the performance of GUMM, we constructed the first ever gene editing scDNAseq “ground truth” atlas which encompasses more than 30,000 cells with known genotype. We created this atlas by expanding several CRISPR-Cas9-edited HL-60 clones, harboring distinct insertion-deletion profiles at select loci, and mixing them at pre-defined ratios to create artificial cocktails that mimic the editing diversity of a CRISPR-Cas9 experiment. These mixtures allow us to explore technical artifacts in the data and optimize our computational workflow in a controlled setting. We show that automated genotyping by GUMM enabled accurate prediction of the original clonal ratios of these sample cocktails. This atlas also serves as a resource for the community to encourage development of computational methods for single cell gene editing assessment. Our study offers both a novel bioinformatic solution and a valuable data resource for gene therapy innovators aiming to expedite the development of engineered cells with intricate genotypes.

P727

A simple and efficient method for specific gene overexpression based on CRISPR activation to develop next-generation MSC-based therapies

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Mesenchymal stem cells (MSCs) possess both immunomodulation and tissue regeneration activities, which are exerted through a wide range of molecular and cellular mechanisms. Indeed, MSCs and their derived extracellular vesicles (MSC-EVs) have demonstrated clinical efficacy in different pathologies including osteoarticular and autoimmune diseases, among many others. Moreover, different modifications have been assayed to increase the therapeutic effects of MSCs, including the use of preconditioning factors and genetic engineering. In this context, overexpression of specific genes or miRNAs in MSCs have demonstrated to improve their cellular properties such as differentiation, migration, homing or survival, as well as to enhance their therapeutic activities.

In this work, we show a simple strategy to specifically overexpress endogenous genes in MSCs by applying CRISPR activation technology. Sequential lentiviral transductions were performed with two vectors, one containing the sequence encoding dead Cas9 fused to the transactivator complex VP64-p65-Rta (dCas9-VPR) and the other containing the specific single-guide RNA (sgRNA) and eGFP sequences. As a proof-of-concept, we focused on the overexpression of endogenous genes from the ULBP family, which are physically close in the genome, thus allowing to prove overexpression specificity. Three different sgRNAs targeting each gene were designed and assayed previously in HeLa cell line, and the one with the best efficiency was selected to modify MSCs. qRT-PCR was applied to assess overexpression of the specific genes, and flow

cytometry was used to determine eGFP protein expression as a readout of transduction efficiency, as well as to analyse membrane expression of the overexpressed ULBP ligands.

Our results showed that at least one of the three sgRNAs was specific in the transcriptional activation of the selected genes (ULBP1, ULBP2, ULBP3 and ULBP6), with more than 3-fold overexpression. qRT-PCR gene expression analyses highly correlated with membrane expression of the ligands determined by flow cytometry. Transduction efficiency in MSCs was higher than 70 % at day one, and eGFP positive cells increased to 98.2 ± 1.9 % (mean ± SD) after antibiotic purification. Gene expression analysis of MSCs modified to upregulate ULBP2 transcription showed more than 5-fold overexpression of the gene, while all the other ULBP ligands did not show significant expression changes, which demonstrates target specificity.

In summary, this method shows high efficiency and specificity on transcriptional activation of endogenous genes. The potential of this approach to overexpress virtually any specific gene, makes this strategy a useful tool not only to screen the effect of specific gene/miRNA overexpression on MSC and MSC-EVs properties, but also to develop next generation advanced or cell-free medicinal products by enhancing the therapeutic activity of MSCs.

P728

Application of Prime Editing gene editing technology as a possible treatment for Autosomal Dominant Polycystic Kidney Disease

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Autosomal dominant polycystic kidney disease (ADPKD) is the most common inherited kidney disease, with a prevalence of 1 in 1000 live births. It is characterized by the appearance of cysts in the kidneys and by an increase in the size of the kidneys, leading irrevocably to end-stage renal disease.

Most of the mutations observed in the genes that cause this disease (*PKD1* and *PKD2*) are missense mutations, which makes possible the application of Prime Editing technology to correct these mutations. The correction of the ADPKD-causing mutation should allow a reversion of the ADPKD phenotype, since it has been demonstrated that the kidney is an organ that has plasticity, that is, the correct re-expression of the *Pkd1*and *Pkd2* genes in mouse cystic kidneys results in a rapid reversal of ADPKD.

The overall objective is to use Prime Editing technology to correct the ADPKD-causing mutation in two different mouse models . The specific objectives are: 1) *In silico* design of pegRNAs; 2) *In vitro* testing of pegRNAs in mouse embryonic fibroblast (MEFs); 3) Bioinformatics study and selection of the pegRNAs with the highest editing efficiency; 4) *In vivo* editing of the two mouse models with the selected pegRNAs.

After *in silico* design of pegRNAs and isolation of MEFs, several *in vitro* nucleofection experiments with different amounts of pegRNAs and Prime Editing plasmids have been performed in these cells. So far, the editing efficiency achieved is low.

New transfection experiments using mRNA instead of plasmid are being carried out to increase the editing efficiency.

P729

Restoration of *ex vivo* telomere homeostasis in dyskeratosis congenita-like CD34⁺ cells through lentiviral gene therapy

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Impaired telomere maintenance is the underlying cause of Telomere Biology Disorders (TBDs). These disorders are primarily characterized by critically short telomeres, which restrict the replicative potential of cells and hinder tissue regeneration and renewal. Complications of TBDs affect multiple organ systems, resulting in organ failure and fibrosis, and imply an increased risk of cancer. Dyskeratosis congenita (DC) is the most common telomeropathy and is considered the prototype of TBDs. Progressive bone marrow failure (BMF) is a life-threatening complication observed in up to 80% of DC patients. Classical DC is caused by germline mutations affecting genes involved in telomere maintenance, with both autosomal and X-linked inheritance patterns. Hematopoietic stem cell transplantation (HSCT) is the current treatment approach for addressing BMF in these patients. However, the poor outcome associated to HSCT in DC patients imply that there is an urgent need to investigate alternative treatment modalities, particularly in the management of BMF in DC patients. To evaluate the efficacy and safety of lentiviral vectors expressing genes associated with the maintenance of telomere homeostasis, we initially generated CRISPR/Cas9-mediated DC-like CD34⁺ cells harboring mutations in the *DKC1* and *TERC* genes. Subsequently, we assessed the telomere length and replicative potential after *in vitro* culture, in both untransduced and transduced DC-like CD34⁺ cells, using *DKC1*- and *TERC*-LVs. Compared with healthy CD34⁺ cells, DC-like CD34⁺ cells showed accelerated telomere attrition. However, transduction with therapeutic vectors improved the telomere length dynamics and the *ex vivo* expansion of DC-like progenitor cells, suggesting the therapeutic potential of these gene therapy approaches for DC patients. Additionally, our data raise a number of questions about the safety of LV-mediated gene therapy in DC. Therefore, current *in vivo* studies are ongoing with the aim of evaluating the efficacy and safety of DC gene therapy in the long-term using *in vivo* animal models.

P730

Large knock-in in primary T cells with optimized Cas9 HDR methods and design

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Knock-in of large transgenes in primary T lymphocytes has previously relied on methods that insert via random integration, not allowing for precise editing and making the genome susceptible to insertional mutagenesis (off-target effects, chromosomal rearrangements, translocations, etc.). As such, there is a need for precision gene editing for introduction of stably expressed transgenes such as chimeric antigen receptor (CAR) variants. CRISPR-Cas9 is an expedient tool for precision editing and can mediate targeted HDR-based insertion. Here we present optimized reagents, donor design, and delivery methods for CRISPR HDR in primary T cells with chemically synthesized short HDR donors for small inserts and PCR-based long HDR donors for knock-in of up to 2kb. These methods and reagents together in primary human T cells achieve high-efficiency precise genome editing with low off-target integration.

P731

The Cells Below: tackling lung basal cells' encasement with VP22 gene editing constructs

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Cystic Fibrosis (CF), is a common autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, encoding for the CFTR ion 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 gene therapy for lung diseases still faces major challenges.

Gene editing of the progenitor lung basal cells (BC) may have the potential to permanently correct any CF causing mutation in the *CFTR* gene. However, whilst luminal epithelial cells (LEC) can be readily transfected with DNA or RNA molecules encoding gene editing constructs, the composition of the LPE causes an impediment to the direct targeting of BC, as efficient delivery of DNA or RNA is mainly limited to LEC. Considering this, is it then possible to deliver a gene editing cargo to the LEC that could then transfer its encoded gene editing protein to the nuclei of BC cells below?

Inspired by previous studies using HSV-1 VP22 cell penetrating peptide (CPP), here we describe the construction of VP22 fusion proteins with intrinsic nuclear localization and intercellular trafficking capacity, using HEK293T cells and a pseudostratified mucociliary epithelium cell model differentiated from BCi-NS1.1. With this approach we will assess if this CPP can shuttle gene editing complexes produced in LEC to the nuclei of BC and functionally correct CF-causing mutations.

Expression vectors encoding for fusion proteins VP22-GFP and VP22-ABE8e(NG) were developed by designing gBlocks encoding for a VP22 sequence and cloning them under control of a CMV promotor in an expression vector (IDTdna.com). HEK293T transfected with pVP22-GFP vectors displayed high levels of GFP expression when measured by flow cytometry 48 hours post-transfection and a distinct nuclear localisation pattern was observed through fluorescence microscopy, while transfection with the pCMV-GFP control vector displayed high levels of GFP throughout the entire cell. To assess the editing capacity of VP22 gene editing constructs we developed an mCherry reporter plasmid containing a premature terminating codon (PTC) in place of Q47. HEK293T cells were co-transfected with pVP22-ABE8e(NG), pmCherry-Q47X and a gRNA plasmid capable of targeting the modified region. Fluorescence microscopy was used 72h post-transfection to detect the red fluorescence of the mCherry reporter as an indicator of the PTC correction. DNA extraction and PCR amplification of the region of interest was carried out to assess the A-to-G editing levels of pVP22-ABE8e(NG).

The current focus is now to determine the intercellular trafficking capacity of these fusion proteins in fully differentiated air-liquid interface cultures of BCi-NS1.1 cells. Seventy-two hours post-transfection, protein localization of VP22-GFP and VP22-ABE8e(NG) will be assessed through immunofluorescence under confocal microscopy, using anti-KRT5 and anti-beta-4 Tubulin antibodies to identify basal and ciliated cells in this model. Fluorescence-activated cell sorting (FACS) will be carried out to sort for BC, allowing to compare the cells' editing level with VP22 gene editing constructs *versus* standard base editors. With this approach we will be able to determine the efficiency of VP22-ABEs to transfer and edit neighbouring cells.

P732

Empower CRISPR by synthetic gene editing

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The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) was harnessed for programmable gene editing in 2012 and have in the last decade revolutionized our abilities to manipulate the genetic code in our genomes. Mediated by a small guide RNA (so-called sgRNA or gRNA), the sole Cas9 protein is directed to the target site in the genome of any species based on the principle of complementary RNA and DNA base pairing between the gRNA spacer and the targeting DNA strand when anchored in the protospacer adjacent motif (PAM). It is thus not surprising that the efficiency and specificity of the CRISPR-Cas9 is strongly depending on the context of the gRNA spacer and PAM sequences. To better understand features affecting CRISPR efficiency and specificity, as well as to develop better CRISPR prediction tools, we have successfully generated large-scale CRISPR on-target and off-target activity data in cells using synthetic and self-targeting CRISPR libraries. Collectively, we define these approaches as synthetic gene editing, which is based on capturing the CRISPR-induced editing in the synthetic and genomically integrated target sites.

The concept of synthetic gene editing is based on synthetic surrogate DNA. Such surrogate is a piece of synthetic DNA which contains the same sequences as the corresponding genomic on-target or off-target site, as well as the protospacer sequences, PAM, and short flanking sequences to capture the local DNA context and CRISPR footprints. Based on the observation that there is a highly concordant editing rate between the surrogate site and endogenous genomic site in the same cell, surrogate reporter vectors have been developed as conventional tools to

quantify the efficiency of gene editing tools and to enrich edited cells with desired mutations. Combined with high-throughput DNA synthesis and DNA sequencing technologies, it is now possible to perform large-scale analysis of CRISPR editing footprints, e.g., indels and modifications introduced to both on-target and potential off-target sites. We foresee that synthetic gene editing will continue to act as an important and high throughput approach to empower the development and application of CRISPR gene editing technology.

P733

A novel method for rapid engineering of hPSCs, compatible with downstream differentiation

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One of the main limitations of Cell Therapies is the manufacturing consistency and cost, hence research on allogeneic 'off-the-shelf' therapies is conducted by both companies and academic institutions around the world. Creating a hypoimmunogenic product, as well as boosting the therapeutic potential of the cells, will often require extensive genetic engineering, including gene knockout an transgene knock-in.

Despite progress in the field brought about by the CRISPR/Cas9 technology, cell engineering still relies on long and tedious procedures, preformed sequentially, to achieve the desired genotype. Transgene knock-in can prove particularly difficult to achieve. Prolonged passaging or differentiation often results in epigenetic silencing of the desired transgene. These setbacks limit the throughput and reproducibility during the generation of cells.

Recently, we published a revolutionary tool for cell engineering called Xential (recombination (X) at a conditionally essential locus), which provides a solution to the aforementioned challenges. Based on Cas9-mediated DNA knock-in, the method allows to insert a gene into a specific locus and effectively select for it in a pool of cells. At the same time, the method enriches for cells where concurrent modifications, such as gene knockout, took place.

I plan to present how, using Xential, one can generate pools, rather than picking clones, of human Pluripotent Stem Cell lines harbouring the desired modifications. Importantly, we continue to observe the expression of our knocked-in transgene after passaging, freeze-thaw cycles and a multistep differentiation procedure into a therapeutically relevant cell type, all in the absence of the initial selective pressure.

P734

Elimination of infectious HIV DNA by CRISPR–saCas9

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The CRISPR-Cas system is a powerful tool for genome engineering that enables the induction of breaks at specific sites. It has been demonstrated that the CRISPR-Cas9 system can be used to inhibit human pathogenic viruses, including the human immunodeficiency virus (HIV). In a previous study, we demonstrated that CRISPR-spCas9 treatment against the provirus can cure HIV infection in T cell culture. However, the large transgenic expression cassette of spCas9 limits its use in gene therapy when using viral vectors with a limited packaging capacity such as AAVs. In this current study, our aim was to identify a Cas system with a smaller expression cassette that could still provide strong resistance to HIV.

We have designed CRISPR-saCas9 gRNAs for highly conserved HIV-1 sequences with no apparent match to human genes to avoid off-target effects. Oligonucleotides encoding HIV-1 targeting gRNAs and control gRNAs were ligated into the pLenti-saCas9 vector. The effectiveness of saCas9 systems with different gRNAs was evaluated by transiently transfecting them with a plasmid encoding pLAI, which encodes the HIV-1 subtype B isolate LAI in HEK293T cells. Candidates with relatively high inhibition of the replication of HIV LAI were selected. SupT1 cells were transduced with lentiviral vectors encoding the chosen CRISPR-saCas9 gRNA candidates and sorted by flow cytometry. Sorted cells were infected with HIV, and virus replication was assessed by scoring syncytia formation.

SaCas9 has demonstrated significant potential in curing HIV infection, with superior performance compared to spCas9. Transduced cells infected with varying doses of the virus were able to be cured by SaCas9 with a single gRNA. However, in some cultures, escape still occurred, particularly at high HIV doses (MOI: 10). Dual-gRNA guided SaCas9 treatment functionally cured all HIV-infected cells. The treatment induced both deletion and insertion at the target site, with the deletion becoming progressively larger over time, leading to the cure of HIV in T cells. These results underscore the potential of SaCas9 as a powerful tool for curing HIV.

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P735

CRISPR/Cas9-mediated base editing efficiently repairs Limb Girdle Muscular Dystrophy type 2A causing mutations

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Limb girdle muscular dystrophies (LGMD) are progressive and rare genetic disorders of which LGMD2A/R1 is the most frequent. LGMD2A is caused by mutations in *CAPN3*, the gene encoding the cysteine caspase 3, and characterized by progressive muscle weakness and atrophy, leading to

loss of ambulation. There is no treatment. More than 600 mutations in *CAPN3* have been described with varying prevalence. The most common LGMD2A causing mutation is *CAPN3* c.550delA, with a prevalence in 1 in 144,000. In our cohort, we have patients carrying several LGMD2A-causing mutations. CRISPR/Cas9-based adenine base editing (ABE) enables the precise targeted conversion of adenine into guanine and is considered a safe tool. We aimed to establish *ex vivo* base editing in G:C>A:T mutations in *CAPN3*.

We selected two base editable *CAPN3* mutations: *CAPN3* c.245 C>T and *CAPN3* c.1469G>A based on their relative frequency among LGMD2A patients and the availability of primary muscle stem cells (MuSC) or induced pluripotent stem cells (iPSCs) from these patients. Using ABE8e-SpRY and an NRN-PAM on the antisense strand, we are able to repair 93% of the *CAPN3* c.245C>T in MuSCs. *CAPN3* c.1469G>A is targeted in iPSCs using the appropriate sgRNA selected after a screen together with ABE7.10 using the NGG PAM. Here, the repair efficiency of *CAPN3* c.1469G>A is 100%. Of note, the targeting sequences carry additional adenines within the ABE activity window and bystander editing is lower than 30%. We have generated a humanized dystrophic mouse model to carry our editing strategy to *in vivo*. In conclusion, base editing in CAPN3 mutations is feasible but requires optimization and our humanized dystrophic mouse model is extremely valuable before clinical application becomes reality.

P736

Permanent ex-vivo rescue of VWF phenotype in VWD type 3 models

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Von Willebrand Disease (VWD) is a commonly inherited and complex bleeding disorder due to the varying forms of the disease. The varying types of VWD result in abnormal von Willebrand Factor (VWF) function and or levels; impacting the effectiveness of primary haemostasis during platelet plug formation. VWD type 3 is classed as the most severe due to absent or undetectable VWF in circulation, which also often impacts factor VIII levels. Patients with VWD type 3 do not respond to common treatments such as Desmopressin (DDAVP), and instead rely on VWF replacement therapies and factor concentrates, which require ongoing monitoring and treatment. Gene therapies such as CRISPR-Cas9 are powerful tools that can be utilised to correct pathogenic mutations to restore healthy VWF function and levels.

The robust nature of the CRISPR-Cas system means it can be tailored to patients with all forms of the disease. Presented here is a previously identified canine VWD type 3 *ex-vivo* model that harbours a homozygous ΔC deletion in exon 4 of the pro-peptide. Homology directed repair (HDR) is one of the cellular DNA repair mechanism, but in combination with a CRISPR-Cas9 Ribonucleoprotein (RNP) and exogenous DNA, this system can be utilised to induce precise

changes at the nucleotide level to correct pathogenic mutations and restore healthy VWF levels and function.

Endothelial colony forming cells (ECFCs) have been successfully isolated and expanded from canine model peripheral blood. Isolated cells have been confirmed positive for multiple endothelial markers, including $CD144^{+}$ / $CD31^{+}$. The genotype and VWF phenotype of the model have been confirmed. Gene editing experiments are currently underway for testing and selecting optimal gRNAs, utilising an in house produced Cas9 protein.

P737

Systematic genomic safety assessment of CRISPR-based gene therapy in human 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. To this date, no medication has been approved for intervention. To lay the groundwork for muscle gene therapy, 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, in agreement with the German regulatory authorities and within the scope of a clinical phase 1/2a study, a systematic evaluation of genome integrity in gene-edited MuSCs. As part of this evaluation, we established GUIDE-seq in MuSCs using an sgRNA of low specificity. We then identified, with high sensitivity, 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 that one candidate sgRNA caused a frequent off-target mutation in the tumor suppressor gene *CUL9*, whereas the other sgRNAs showed a safer profile. These results underline the importance of a thorough genetic safety assessment, and are now complemented with deeper genome integrity analysis to exclude large scale genomic aberrations.

P738

Nickase deficient ABE8e efficiently edits paralogous *HBG* genes without deletion of intervening 4.9kb region

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Reactivation of fetal hemoglobin is the most promising curative approach for beta hemoglobinopathies. Recreating HPFH (hereditary persistence of fetal hemoglobin) mutations or

introducing novel mutations in the promoter region of gamma globin is a widely accepted method for therapeutically elevating fetal hemoglobin without affecting other cellular activities. CRISPR/Cas9 based editing of the gamma globin promoter has been demonstrated by several groups previously. However, due to the sequence similarity between the homologous A gamma and G gamma (*HBG1* and *HBG2*) genes, the intervening region between the two gRNA binding site (4.9kb, including the HBG2 gene) gets deleted when editing with Cas9 nuclease. Base editing in gamma-globin promoter is a promising approach for reactivation of fetal-hemoglobin without causing double strand break. Previously, by an arrayed screening we had identified several gRNAs that can be utilised for therapeutic elevation of fetal hemoglobin. However, we had noticed that base editing, although to a lesser extent than Cas9, causes 4.9kb deletion of the intervening region due to high homology between *HBG1* and *HBG2* genes even without double strand break due to the presence of DNA nicks. Here we sought to investigate whether the deletion frequency is gRNA dependent and if the use of nickase deficient dCas9 Adenosine Base Editor can prevent the generation of large deletions. We performed a comprehensive evaluation of the editing outcome and frequency of large deletion using dCas9, nCas9, dCas9-ABE8e and nCas9-ABE8e in the gamma globin promoter. We found that while nicking in itself induced large deletions, the frequency reduced upon efficient base editing. There was no appreciable deletion with the use of dCas9-ABE8e making it a safer approach, in terms of genome integrity, for therapeutic genome editing in the gamma-globin locus. Overall, this study would be an important next step in increasing the safety of genome editing.

P739

Detection and removal of cryptic dsDNA in ssDNA homology donor preparations

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Site-specific edits to the genome of CD34+ Hematopoietic Stem Cells (HSCs) can be made by a combination of targeted double-strand breaks using CRISPR/Cas9 and incorporation of a Homology-Directed Repair Template (HDRT), a DNA template provided in *trans* that encodes the desired edit within a sequence homologous to the sequences around the cut site. There is increasing interest in using non-viral HDRTs in order to avoid the genotoxicity, p53 activation, loss of engraftment potential and manufacturing costs associated with some viral templates. ssDNA Homology-Directed Repair Templates (HDRTs) have been found to be superior to dsDNA templates for site-specific CRISPR-mediated knock-in of genetic constructs due to reduced toxicity. However, some toxicity is still observed.

A variety of methods exist to generate ssDNA HDRTs, the most commonly used of which is Streptavidin capture of dsDNA generated by PCR with one biotinylated and one non-biotinylated primer, followed by strand melting and ssDNA elution with NaOH. Some dsDNA is observed to elute alongside the ssDNA due to Streptavidin denaturation, but it has been previously reported that this can be minimised by reduction of NaOH concentration and pre-incubating capture microbeads. We found that in addition to dsDNA, substantial quantities of undesired strand elute and remain as ssDNA after neutralisation before re-annealing during downstream processing and use, increasing dsDNA contamination. We found that while existing mitigation strategies appeared to effectively reduce the initial appearance of dsDNA, they did not substantially reduce contamination as revealed by deliberate re-annealing of cryptic dsDNA. We therefore developed a

Re-Anneal and Digest (RAD) protocol which can be readily incorporated into the existing isolation workflow, which reduces undesired strand and dsDNA to undetectable levels while maintaining a good yield of desired ssDNA and could prove advantageous in safeguarding the functionality of HSCs undergoing gene editing.

P740

Base editing for C-terminal dominant mutations in Rhodopsin gene

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A common cause of blindness is represented by mutations in the Rhodopsin (*RHO*) gene, accounting for 25% of autosomal dominant Retinitis Pigmentosa (adRP). Missense variants at C-terminal proline 347, such as p.Pro347Ser (c.1039C>T, P347S-RHO) or p.Pro347Leu (c.1040C>T, P347L-RHO), are among the most recurrent RHO pathogenic variants in Europe, affecting the post-Golgi transport to outer segments (OS). We previously showed allele-specific knockout of *RHO* carrying a C-terminal P347S mutation with the CRISPR/Cas9 system *in vitro* and in a mouse model of adRP demonstrating restoration of the correct localization to the OS in AAV-CRISPR treated mice. Here, we propose to use adenine base editors (ABE) to correct the P347S-RHO or P347L-RHO without inducing DNA double-strand breaks (DSB). We designed strategies based on NG-ABE8e, SpRY-ABE8e or Nme2-C-ABE8e to specifically target RHO mutations, with limited risk of perturbing the coding sequence. We screened base editors *in vitro* in HeLa clones stably expressing P347S, P347L or wt *RHO*, and transfected with effector plasmids for base editors, to assess the best platform to achieve correction of the mutated nucleotide. Frequency and specificity of base editing were assessed by BEAT analysis and NGS, and showed that NG-ABE8e was highly efficient in correcting RHO C-term mutations. No bystander A-to-G conversions in nearby nucleotides were observed, preserving the RHO coding sequence. Ongoing analyses are evaluating base editing efficiency on hTERT-RPE1 cells overexpressing P347S-RHO or P347L-RHO fused to a reporter gene, which will also represent a cell model for the comprehensive characterization of the subcellular localization of RHO variants. Our results will provide clear evidence about the employment of adenine base editors to repair C-terminal dominant RHO mutations and paves the way for the permanent and precise correction of heterozygous variants in dominantly inherited retinal diseases.

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P741

CRISPR-Cas mediated base editing approaches for CRB1 related retinal dystrophy

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Pathogenic variants in *Crumbs homolog 1 (CRB1)* gene lead [1]to severe, childhood-onset retinal degeneration leading to blindness in early adulthood. There are no approved therapies and traditional adeno-associated viral vector-based gene therapy approaches are challenged by the size of the *CRB1* which exceeds the vector carrying capacity and the existence of multiple CRB1 isoforms. Here we describe 3 *CRB1* variants, including a novel previously unreported variant, which led to retinal degeneration and offer a CRISPR-Cas mediated DNA base editing strategy as a potential future therapeutic approach.

Retrospective case series. Clinical and genetic assessments were performed, including deep phenotyping by retinal imaging. In silico analyses were used to predict the pathogenicity of the novel variant and determine whether the variants are amenable to DNA base editing strategies.

Case 1 was a 24-year-old male with cone-rod dystrophy and retinal thickening typical of CRB1 retinopathy. He had relatively preserved central outer retinal structure and best corrected visual acuity (BCVA) of 60 ETDRS letters in both eyes. Genetic testing revealed compound heterozygous variants in exon 9: c.2843G>A, p.(Cys948Tyr) and a novel variant c.2833G>A, p.(Gly945Arg) predicted to be pathogenic by in-silico analysis. Cases 2 and 3 were two brothers, aged 20 and 24, who presented with severe cone-rod dystrophy and significant disruption of outer nuclear layers. BCVA was reduced to hand movements in Case 2 and 42 ETDRS letters in Case 3. Case 2 was also affected with marked intraretinal fluid, common in CRB1 retinopathy, but responded well to treatment with oral acetazolamide. Genetic testing revealed two c.2234C>T, p.(Thr745Met) variants in both brothers. As G-to-A and C-to-T variants, these all three variants are amenable to adenine base editors (ABEs) by targeting the forward strand in Case 1 variants, and the reverse strand in Cases 2 and 3. Available PAM-sites were detected for KKH-nSaCas9-ABE8e for the c.2843G>A variant, nSaCas9-ABE8e and KKH-nSaCas9-ABE8e for the c.2833G>A variant and nSpCas9-ABE8e for the c.2234C>T variant.

In this case series, we report three pathogenic CRB1 variants, including a novel c.2833G>A variant, associated with early-onset cone-rod dystrophy. We highlight the severity and rapid disease progression and offer ABEs as a potential future therapeutic approach for this devastating blinding condition.

P742

Gene editing for RYR1-related myopathies

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Gene therapy is a promising approach to treat genetic diseases such as congenital myopathies. Gene replacement strategies have already been developed for other myopathies like Spinal Muscular Atrophy (SMA) and Duchene Muscular Dystrophy (DMD). For diseases associated with mutations in the *RYR1* gene (*RYR1*-related myopathies), the size of the gene limits its insertion into a therapeutic vector. Therefore, gene editing seems to be the ideal strategy and especially with CRISPR/Cas9. Several strategies can be used to introduce gene editing tools into muscle cells. Current methods use viral vectors, which are largely effective but are limited by prolonged Cas9 expression, increasing the risk of off-target cleavage. On the other hand, non-viral techniques are increasingly being developed to safely transduce target cells, but they have not been extensively studied in muscle cells. The aim of our study is to compare different viral and non-viral approaches to deliver Cas9 protein and *RYR1*-targeted guides RNA (gRNA) into muscle cells in vitro. For this proof of concept, we chose to delete a *RYR1* sequence, resulting in a knockout (KO) and to further characterize the cells at the functional level (Ca^{2+} release). It has been shown that *RYR1* KO cells are unable to release calcium (Ca^{2+}) from the sarcoplasmic reticulum upon stimulation. Therefore, assessment of Ca^{2+} release by Ca^{2+} imaging in edited cells is a reliable readout of gene editing efficiency. We compared four methods of Cas9-gRNA delivery in human immortalized muscle cells : lentiviral vector-based transfer of Cas9 and gRNA, transduction with muscle-specific AAV vectors, electroporation of Cas9-gRNA ribonucleoproteins (RNPs) and nanoblades, a recently developed non-viral approach based on virus-like particles. The latter is a good candidate to combine the efficiency of viral transduction with the absence of DNA transfer into the host cell. This study will allow the development of therapeutic approaches for *RYR1*-related myopathies by using the most relevant and efficient strategy for Cas9 delivery.

P743

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 3 cell lines. With these enormous amounts of data generated, it will strongly aid the better development of deep learning models for choosing the best SpCas9 variant for given targets, thus further advancing the CRISPR-based applications in basic and clinical research.

P744

Efficient and gentle non-viral engineering of iPSCs by photoporation

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Human induced pluripotent stem cells (iPSCs) have revolutionized regenerative medicine due to their ability to differentiate into any cell type and propagate limitlessly. Engineered iPSCs are a promising excellent source for the production of cells for cancer immunotherapy, e.g. (off-the-shelf) iPSC-derived CAR-NK or CAR-T cells. Additionally, gene-edited patient-derived iPSCs pave the way for the treatment of genetic disorders in an autologous setting.

Unfortunately, the many promising applications of gene-edited iPSCs have not yet been able to reach their full potential, among other due to suboptimal transfection technologies. While the search for better delivery methods has gone on for decades, none of them are fully satisfactory. As viral and non-viral nanocarriers struggle with respectively safety concerns and limited efficiency, interest in physical delivery methods has witnessed a steady increase in the past decade. Electroporation is a well-known physical transfection technology, yet it is notorious for its detrimental effects on cell viability and functionality. Photoporation is an up-and-coming alternative intracellular delivery method which has gained interest due to its gentleness to cells. The modality is based on the combination of laser light and photothermal nanoparticles to create transient pores in the plasma membrane, thus allowing uptake of external macromolecules. In its most traditional form, cells are first incubated with photothermal nanoparticles, e.g., gold nanoparticles, which can adsorb to the cell membrane. Local heating effects upon laser irradiation create pores in the membrane through which compounds in the surrounding cell medium can enter the cell.

To facilitate clinical use, biodegradable and biomimetic polydopamine photosensitizers form an alternative to the more common non-degradable gold nanoparticles. Alternatively, the photothermal nanoparticles can be safely embedded in nanofiber substrates onto which cells can be cultured, transfected and recollected without direct contact between cells and nanoparticles. Here we evaluate and fine-tune both photoporation modalities for the transfection of sensitive iPSCs.

P745

Directed integration of a transgene into a safe-harbor site using non-integrating lentiviral vectors and nickingCas9

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In the last decades, gene editing has been a topic of interest and a milestone in this field was the identification of the CRISPR-Cas (Clustered Regularly Interspaced Short Palindromic Repeats and Cas for CRISPR associated protein) system. CRISPR-Cas was first identified as a bacterial defense mechanism against bacteriophages but it's potential in genetic modification was quickly realized and it was adapted for this. The Cas has an endonuclease activity which can cleave double stranded DNA in a specific spot through the complementarity of a guiding RNA (gRNA). Taking advantage of this system, different genome modifications can be made including insertion of DNA templates through homologous recombination. However, efficient delivery of the required components can be limiting, and safety concerns of double stranded breaks have long been recognized. Lentiviral vectors very efficiently transduce different non-dividing and dividing cells and would be suitable delivery candidates. Lentiviral vectors are integrating and so would provide long term expression of the CRISPR-Cas9, which would not be desirable. Subsequently, we have designed a strategy for genetic modification using a non-integrating lentiviral vector(Schenkwein, Turkki, Kärkkäinen, Airenne, & Ylä-Herttuala, 2010) combined with the nicking Cas9(Ran et al., 2013) to avoid double strand breaks. In this system two types of vectors were designed, one containing the nicking Cas9 and a gRNA targeting a safe harbor on Chr1 and the second containing the desired homologous cassette and the second gRNA required to nick the second strand. As proof of principle, the green fluorescent protein (GFP) was flanked with the homologous arms to the targeted harbor site to allow for its recombination at that locus. The efficacy of the recombination was assessed following transduction of both vectors and culturing transduced cells for up to 1 month. Flow cytometry was used to detect fluorescent cells and PCR to amplify the integrated cassette which was then analyzed by Sanger sequencing. Our preliminary results in 293T cells have shown successful targeted integration via homologous recombination using CRISPR-Cas9n, in the context of non-integrating lentiviral delivery.

P746

Exploring the role of the nlgn1 gene in the response to stress through a CRISPR Cas9 nlgn1 zebrafish knockout

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Post-traumatic stress disorder (PTSD) is a global mental health challenge, affecting individuals exposed to diverse sources of trauma. In our study, we aim to understand the role of the Neuroligin 1 (*nlgn1*) gene in the response to stress and the genomic network affected by its deletion, using a zebrafish animal model. NLGN1 is a neuronal cell membrane protein that plays a

role in synapsis formation and remodeling processes. NLGN1 has been associated with PTSD in previous GWAS studies; however, its role in psychiatric disorders including PTSD remain elusive. Using CRISPR- Cas9 genome editing, we are currently in the process of generating a *nlgn1* zebrafish knockout line (KO). We found the *nlgn1* knockout produces strong malformations in development that decreasing survival to less than 40%. We also conducted behavioral tests in F0 to investigate the stress response to acute stress stimuli and found that *nlgn1* CRISPR KO larvae have an increased reaction time and total distance traveled compared to controls. An F1 population which mutated sequence is known, is growing currently and the behavioral test will be conducted in them.

This study is part of a larger translational study which aim is to identify genetic variants that might affect the risk of developing PTSD or that confer resilience in a Colombian population exposed to the Armed Conflict. It holds immense implications for our understanding of PTSD and the advancement of therapeutic approaches. By unraveling the role of *nlgn1* in stress-related disorders, we aim to shed light on the molecular pathways involved in PTSD development and contribute to the understanding of this complex disorder.

P747

Base editing of chronic granulomatous disease-causing mutations in *CYBA* and *CYBB*

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Chronic granulomatous disease (CGD) is an inborn error of immunity resulting from phagocyte dysfunction caused by pathogenic variants in the genes that encode the subunits of the nicotinamide adenine dinucleotide phosphate oxidase complex. CGD can be treated by allogeneic hematopoietic stem cell (HSC) transplantation, however, this carries the risk of severe adverse effects, such as graft-vs-host disease and graft failure. To overcome this, development of *ex vivo* gene editing of autologous human HSCs is being studied. DNA double-strand break (DSB)-independent gene editing technologies such as base editing have been developed to circumvent the challenges of gene editing based on CRISPR/Cas9 and homology-directed repair (HDR), including unintended indel formation, risk of chromosomal rearrangements, as well as toxicity in HSCs. Here, we present ongoing work using base editing to correct CGD-causing mutations in the *CYBA* and *CYBB* genes. We generated clonal K562 cell line models carrying the mutations *CYBA* c.371C>T, *CYBB* c.252G>A, and *CYBB* c.625C>T. Base editing was performed by nucleofection of the K562 clones and in the case of the *CYBA* c.371C>T variant also patient-derived peripheral blood mononuclear cells (PBMCs) with in vitro transcribed base editor mRNA and synthetic single guide RNAs. The adenine base editor ABE8e-NRCH, based on a SpCas9 variant with an NRCH PAM sequence preference, was used for correction of the *CYBB*c.252G>A and *CYBB* c.625C>T variants in K562 cells and resulted in base editing efficiencies of 22% and 96%, respectively. Similarly, base editing efficiency of 99% was obtained for the *CYBA* c.371C>T variant in K562 cells using adenine base editor ABE8e, which is based on

the conventional SpCas9 variant with an NGG PAM sequence preference. In addition, base editing of this variant in patient-derived PBMCs resulted in high on-target editing efficiencies of more than 85% with less than 0.5% indel formation and bystander editing of less than 2%. These results demonstrate the high efficiency and product purity of base editing and support further exploration of base editing for treatment of CGD. Future studies will include base editing of CD34+ hematopoietic stem and progenitor cells (HSPCs) derived from heterozygous carriers of the variants or healthy controls after transduction with lentiviral vectors carrying the gene variant. Furthermore, safety validation of the bystander edits, off-target analyses, and engraftment studies of base edited CD34+ HSPCs will be performed.

P748

Single-Cell DNA Sequencing of Gene-Edited Cells Enables In-Depth Characterization of Cell and Gene Therapy Products

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Advances in cell and gene therapy (CGT) are transforming the way we treat and potentially cure certain diseases including cancer and rare genetic disorders. Many of these therapies involve genetic engineering cells in order to replace missing or malfunctioning genes, or alter existing genes. Gene editing tools like CRISPR have revolutionized how we change an organism's DNA and are proving to be powerful modalities for novel therapies. Despite its ability to precisely alter the genome, gene editing yields heterogeneous populations where some cells may have undesired editing outcomes. Cell-to-cell variation in on- and off-target editing, knockin efficiency and copy number, and the generation of rare chromosomal alterations (e.g. translocations) can impact the safety and efficacy of therapeutic agents.

For this reason, in-depth characterizations are critical to successfully bringing new CGT products to market. Unlike conventional bulk DNA sequencing, single-cell sequencing can achieve high resolution assessment of intended and unintended edits as well as zygosity and co-occurence of alterations within individual cells. Here, we demonstrate a multi-omics platform that analyzes genotype and phenotype simultaneously from single cells, providing sensitive and nuanced quantification of genetic edits made to up to 10,000 individual cells with a simple workflow. The platform allows comprehensive characterization of cells as well as the rapid optimization of gene editing protocols.

The workflow leverages microfluidics technology to link cell-specific barcodes with targets through multiplexed PCR. Streamlined bioinformatics analysis of single-cell sequencing data reveals the distribution and frequency of on- and off-target edits, edit zygosity, edit co-occurrence, and translocations in the pool of edited cells.

To enable characterization of gene editing, including SNVs, short indels (up to 30 bp), large indels (50bp up to 100kb), and translocations, we developed targeted panel design strategies to ensure efficient amplification of targets, and a modular analysis workflow to automate single-cell DNA sequencing analysis and visualization. We then characterized the performance of the assay on cell lines with known truth (SNV and indels) as well as orthogonally validated pools of edited cells. The results demonstrate that the targeted sequencing assay is sensitive, accurate, and precise across a

range of SNV, short indels, and large indels. The assay also reliably co-measures on- and off-targets, zygosity and co-occurrence at single-cell resolution. Together, with the visualization and report automation, our single-cell NGS assay enables rapid optimization of gene editing protocols, guidance for disease modeling, and provides rich characterization of cell-based therapeutics to ensure safety and efficacy.

P749

Synthetic DNA and targeted non-viral delivery systems for genetic correction of Duchenne's Muscular Dystrophy

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Duchenne muscular dystrophy (DMD) is a severe monogenic disease caused by mutations in the gene encoding dystrophin. While several trials are on-going, including AAV-mediated delivery of a truncated micro-dystrophin, no clinically effective therapy is available to date. The emergence of CRISPR-Cas and associated editing technologies provide an attractive therapeutic option for the treatment of DMD, however delivery to muscle remains a challenge.

4basebio has developed a proprietary, non-viral nanoparticle that targets muscle cells with high specificity and low immunogenicity, allowing for repeat dosing. The Hermes™ particle is payload agnostic and has been used to deliver DNA, mRNA and proteins to muscle cells both *in vitro* and *in vivo*.

In addition, we have a scalable enzymatic synthesis process for the production of linear DNA constructs via our Trueprime amplification technology. The oeDNA produced is devoid of any bacterial backbone and the manufacturing process circumvents cumbersome fermentation processes required for plasmid DNA, is size and sequence independent, and ideally suited for DNA repair templates used in gene editing.

In this study, we utilised the muscle targeting particle to deliver Cas9 protein with sgRNAs targeting the DMD mutational hotspots in C2C12 myoblasts and myotubes or Hek293 cells. Particles had highly favourable biophysical characteristics, stability, encapsulation of payload, and achieved high KO efficiency ranging from 23% to 71% in relevant cell models.

To investigate a gene-integration approach using our plasmid-free synthetic DNA technology, we designed two oeDNA™ templates encoding either the cDNA sequence of the *Dystrophin* gene or an EGFP cassette with flanking homology arms. The co-delivery of template DNA along with the RNP complex encapsulated in our Hermes™ nanoparticle resulted in a successful integration of the oeDNA template, and enhanced protein expression *in* vitro. Mouse studies confirming these findings are ongoing. However, preliminary data showed a 10-fold increase in the expression of luciferase in the muscle compared to liver for our Hermes™ nanoparticle.

The present study provides proof-of-concept for the use of Hermes™ particles to deliver RNP + donor templates to reframe or fully replace a dysfunctional gene, offering a promising approach for DMD therapy.

P750

Investigation of tRNA and Ribozyme-based cassette, driven by the pol-II polymerase for PE3 prime-editing

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Owing to low efficiency of prime-editing(PE), any modality toward enhancing the efficacy and specificity is of great importance. In this study, different *polII* driven cassettes were compared for their editing efficiency.

First, pegRNA specific cassette targeting VEGF-A and HEK3 locus, to introduce or delete 3 nucleotides into/from HEK3 and VEGF, were designed with surrounding ribozyme or tRNA. The pegRNA cassette and sgRNAs were synthesized, cloned into the GFP expressing plasmid and analyzed by fluorescent microscopy and qPCR. After plasmid transfection, cells were harvested, and genomic DNA were introduced into separate in-house ddPCRs to compare event calls. Then, the amplified genes were investigated for intended edit and bystander mutations through Amplicon deep-sequencing.

The gene expression analysis result showed a suitable expression pattern of cassettes. No sign of dramatic change in cells were observed in short follow-up. The ddPCR methods showed very low detection limit(LOD) for all editions. In contrast to normal sequencing, the ddPCR analysis showed to be very precise and sensitive in revealing variety of editing efficiency between cassette to find deletion/insertion and between U6-driven versus *polII*-driven constructs. The amplicon sequencing analysis was also in agreement with ddPCR data.

Finding a universal cassette suitable for every prime editing circumstance seems to be hard to achieve due to its context dependency and effects of many other factors. While tRNA and ribozyme cassette served as a suitable frame for PolII-driving construct, they need to be optimized for each editing purpose.

P751

Enabling GMP production of sgRNA for CRISPR-based cell and gene therapies

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Synthego supports the development of CRISPR-based cell and gene therapies, from discovery to first in human clinical trials. We have developed automated platforms for synthesizing CRISPR guide RNAs and engineering cell lines at scale to support preclinical research. Here, we present our work to launch a new, state-of-the-art GMP facility in mid-2023 that is specifically designed to support scientists and clinicians in with clinical-grade CRISPR sgRNA for Phase I clinical trials. In addition, we highlight how we have supported a current CRISPR gene therapy program at an academic medical center, from discovery research to an upcoming clinical trial slated to begin in late 2023.

P752

Optimization and characterization of donor-to-donor variability of primary natural killer cells transfected with RNP

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Cell and gene therapy manufacturing relies on robust and consistent delivery of payloads, such as mRNA, DNA, and CRISPR-Cas RNPs to primary cells. Flowfect® is a novel transfection technology platform (developed at Kytopen, an MIT spinout) that combines continuous fluid flow with electric fields to deliver payloads to cells of interest. Flowfect® provides the benefit of flexibility and customization of transfection parameters for each individual cell process as compared with existing delivery techniques. This customization is performed in a small volume, high throughput device which enables predictive scaling to larger manufacturing volumes.

We have previously demonstrated the optimization of delivery of mRNA and RNP payloads to primary T cells. Here, we focus on the delivery of CRISPR-Cas9 RNP targeting the TIGIT locus to primary Natural Killer (NK) cells. After screening various Flowfect® parameters in expanded NK cells, we generated a Pareto optimization model of the input design topography. We selected three Flowfect Profiles™, unique combinations of adjustable parameters that predicted optimized editing efficiency, post transfection viability, and population doubling levels (PDL), relative to a reference configuration. The performance of these profiles was subsequently tested in three additional donors, comparing efficiency and cell health to a traditional static electroporation process. We found that though all donors showed high knockout of TIGIT protein expression with these configurations (>60%), not all donors had high levels of INDELs by Sanger sequencing and TIDE analysis, indicating that protein knockout may not be a good surrogate for editing efficiency. Ancillary flow cytometry analysis revealed retention of a CD56+/CD16+ population after transfection compared to static electroporation conditions, suggesting retention of mature and cytotoxic NK cell phenotype using Flowfect® compared to static electroporation. We followed up with transcriptome profiling in these donors to understand differences in gene expression. RNA-Seq was performed on each donor at 3 timepoints post transfection. This analysis showed overall lower fold-change in differentially expressed genes 48-hours post-transfection compared to 5-hour and 24-hour timepoints. Analysis using second-generation p-values further clarified practically significant differences in gene expression over time and between transfection conditions. We found that while overall gene families were similar between donors and transfection conditions, specific differentially expressed genes varied between donors at all timepoints. These results provided further insight into the impact of donor-to-donor variability during the transfection optimization process. Transcriptome analysis of multiple donors may help highlight and identify pathways that could be targeted during the manufacturing process to improve transfection efficiency and cell health.

P755

Optimising gene editing by Prime Editing (PE) in hiPSCs and whole brain organoids: exploring the field of nanoparticle-based delivery

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Autism Spectrum Disorder (ASD) is one of the most prevalent neurodevelopmental disorders, reaching over 1% of individuals diagnosed worldwide. Its wide heterogeneity makes it particularly challenging to determine the ASD aetiology or to design a pharmacological treatment. While hundreds of ASD risk genes are being identified, no single one is found in more than 2% of diagnosed individuals, raising the need for a personalised approach. Three-dimensional culture systems, such as cerebral organoids generated from human induced pluripotent stem cells (hiPSCs) hold significant potential to model neurodevelopmental diseases in a complex, tissue-like environment. Organoids can contribute some shortcomings of the two-dimensional model, such as the presence of diverse cell-type progenitor lineages, and several neuronal subtypes which are potentially self-organized to form networks, whose identity can be confirmed through single-cell RNA sequencing -scRNAseq- among other analysis. Cutting-edge genome editing tools are currently overcoming limitations regarding personalized medicine, as can be used to correct disease mutations. The CRISPR/Cas9 system has been widely used to perform gene editing by creating specific double-strand breaks (DSBs) in the genome, which can result in a variety of undesirable outcomes such as insertion and deletions -indels- at the target site, translocations, large deletions, or aneuploidy. Hence, recent studies conducted by David Liu *et al* have pioneered base editing -BE- and prime editing -PE- standing as a versatile and precise methods that directly re-write novel information into the targeted DNA region without creating DSB. Our aim is to create patient-derived hiPSCs *in vitro* models in which ASD aetiology will be investigated and potential gene therapy could be developed according to the detected mutation. First, a study with wild type (WT) hiPSCs is being conducted by introducing an ASD risk mutation through precision gene editing technology, Prime Editing (PE); as gene editing efficiency can vary across enhanced PE systems, all PE2, PE3, PE4 and PE5 updates will be optimised via mRNA delivery to get the higher gene editing efficacy in our cell line. The *knock-in* (KI) hiPSCs will be differentiated into a whole-brain ASD-like three-dimensional (3D) model representing the neurodevelopment of an individual carrying an ASD risk mutation by using adjusted published protocols. Finally, although most of the therapies targeting the brain are being encapsulated in adeno-associated virus -AAV- as brain tropism can be reached, they are also linked to several undesired effects in some patients. Some of the most remarkable effects could be the trigger of an enhanced immune response, potential toxicity when delivered at high doses -moreover, when AAV provides a transient expression, and multiple dose applications are needed-, or risk of insertional mutagenesis which might disrupt some gene functions. Hence, we propose a nanometric delivery system that will encapsulate the PE machinery -*in vitro* transcribed and synthetic mRNAs- penetrating both the 2D and 3D model, attempting to reverse the phenotype displayed in both models. This study could be applied to patient-derived hiPSCs which ultimately could set a precedent for evaluating gene therapy in ASD- derived *in vitro* models.

P756

Targeted genomic insertions with an all-in-one lentivector containing Cas9 protein, sgRNA and donor DNA

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Targeted knock-in of large DNA fragments into genomic loci holds great potential for treating genetic diseases. We have developed a lentivector that carries all the components needed for site-directed insertions, including the Cas9 protein and a template for both the guide RNA and donor DNA. Simultaneous digestion of the genomic and lentivector reverse-transcribed DNA resulted in the insertion of the donor into the target site via the non-homologous end-joining pathway. The 6kb-long promoterless donor, which contained IRES-puroR-P2A-mCherry cassette, was inserted into an EGFP locus in HEK293-EGFP cells as evidenced by the mCHerry expression in ~4% of cells. The proportion of mCHerry+ cells was increased to >90% by growing the cells in a puromycin-containing medium. We confirmed the correct insertion site by junction PCR and the insert length by a long-template PCR.

P757

A new paradigm shift in characterization of genetically transformed cells for Cell & Gene therapy and Biomanufacturing.

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The development of recombinant DNA technology to modify genetic material has paved the way towards target driven alteration within the desired genome for multiple applications, majorly in healthcare and clinical therapeutics. Using such technology, domain of cell & gene therapy (CGTs) has advanced medicinal products to treat severe and rare diseases. While, gaining traction to advance rapidly towards personalized precision medicines, this field has multiple challenges primarily regarding patients safety. Some of these risks can be mitigated by increasing the control of such products at very early stages to avoid any hazardous effects by dysregulating other functions within modified cells. Presently, the CGTs and biomanufacturing industries are obliged to use combination of multiple technologies at different levels to assess and check engineered genome modifications. To substitute such dependencies towards multiple technologies and reduce turnaround time and cost; Molecular Combing technology, provides the prospect as the tool for characterization of genetically transformed cells.

Molecular Combing Technology (MCT), where millions of individual DNA molecule are stretched uniformly on a solid surface, facilitates the direct physical identification and measurements of target region (‘gene of interest’) within the model cell line/organism genome (via specific florescent probes design).

With MCT, we introduce the 6 parameters in 1 assay, a as unique tool for highly specific identification & characterization of genetic material, for evaluating:

- Genetic stability & productivity
- Genetic integrity
- Clonality

The 6 parameters are:

- Integration Identification.
- Copy number determination.
- Orientation assessment.
- On & Off target insertion.
- Genetic Integrity of Target region.
- Genetic Signature for stability assessment.

Based on our AI-based analysis software, we generate statistical plots in form of reports, to facilitate quantitative decision making power for the experts. Thus, with MCT we envision to provide solutions towards reduction in turnaround time & development cost as a one stop shop solution for the industry.

P758

Cell line development for optimised cultured meat production

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Cell line development for cultured meat production is a relatively new area, and one which can learn substantially from the cell and gene therapy field. Engineering of animal-derived cell lines for desirable traits, such as immortalisation, rapid proliferation capacity, enhanced differentiation, and suspension culture, is critical from a process and cost perspective. Notably, regulatory and consumer perceptions are influenced by the specific types of cell modifications, and the methods used to generate them, with precise mutations engineered using transiently expressed base- and prime-editing techniques likely to be preferred. Genetic and phenotypic stability over numerous population doublings is required to produce the substantial biomass required for meat production. We will present several proof-of-principle results from our cell line development pipeline, including immortalised bovine myogenic and adipogenic cell lines carrying modifications to a variety of target genes, including p16, TERT and CDK4, and adipogenic cells that can be inducibly differentiated into muscle through expression of MyoD. We will present further ideas and concepts for the design and construction of bioprocess-optimised cell lines, and are looking forwards to ideas and feedback from the wealth of CGT expertise present.

P759

Generation of a universal viral vector production cell line – OXBHEXs

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The integration of the Simian Virus 40 T antigen into the Human Embryonic Kidney clone 293 (HEK293) resulted in the generation of the widely used HEK293T cell line. In contrast with the parental HEK293 cell line, HEK293T cells display several advantageous attributes, namely increased growth kinetics, high transfectability and superior lentiviral vector (LV) yields. To support the large-scale production of LV and to meet clinical demands, Oxford Biomedica (OXB) has in addition established a HEK293T cell line adapted to serum-free suspension growth conditions (1.65s cell line). Similar HEK293 suspension cell lines exist which support the large scale production of other viral vectors systems, such as AAV and Adenoviral vectors. The ideal scenario would be the creation of a universal HEK293-based cell line that could support production of all viral vectors (LV, AAV and Adenovirus).

We generated cell lines using genome editing to knock-out the SV40 T antigen from the 1.65s cell line. Nanopore sequencing of this region identified two copies of the target gene flanking a neomycin resistance (NeoR) cassette. To create a large sequence deletion, crRNAs targeting SV40 T antigen and the NeoR were used. Subsequently, clones were isolated and selected from the edited pool of cells resulting in a number of candidate clones, henceforth known as OXBHEXs. Nanopore sequencing performed on the best candidate clones confirmed the indels leading to the knock-out of the SV40 T antigen gene. Additionally, Western blot and Mass Spectrometry analyses showed absence of the SV40 T antigen protein/peptide sequences.

The clones were screened based on viral vector productivity, sensitivity to Geneticin and absence of any detectable SV40 T antigen peptides. In addition, Nanopore sequencing was used to verify any deletions encompassing the SV40 T antigen coding regions as well as the Neomycin resistance gene. A clone was identified that was particularly attractive due to a large deletion that resulted in the lack of any intact coding sequences for either the SV40 T antigen or Neomycin resistance genes with high LV production capability. This new cell line offers a number of advantages for production of viral vectors but also for the development of new stable basal, packaging and producer cell lines.

In summary, the newly generated OXBHEXs cell line presents a safer, more universal and streamlined approach in the development of life-changing gene therapy viral vectors.

P760

The LumiPoreTM, non-viral cell transfection platform Photoporation: high viability, high throughput rate of payloads delivery

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Trince's LumiPore™ cell transfection platform, combines laser exposure with photothermal nanoparticles to convert light energy into heat to transiently permeabilize the cellular plasma membrane. It can be used to deliver a wide variety of effector molecules (e.g. nucleic acids or proteins) into the cytosol of any cell type, adherent and suspension, including hard-to-transfect cells such as immune cells for cell-based therapies.

The technology, called photoporation, offers a high-throughput rate with an 96-well plate which contains 100Million T cells photoporated in less than 10min. It also ensures that the cells maintain their proliferative and functional properties post photoporation, maximizing the therapeutic quality of the final cell product.

This method is harmless to cells, cells do not need recovery days post photoporation, neither media change or, cells' detachment from their culture support prior to photoporation (if adherent cells).

The LumiPore™ is a benchtop instrument, compatible with well-plates, T-75 flask and, as a unique advantage allows the possibility of spatial-selective intracellular delivery in cell subpopulations within a culture, even with single-cell resolution or, in a specific area of the well.

P761

Assay development for phagocytosis activity evaluation

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The increased risk of developing age-related diseases in the elderly is associated with an imbalance and deficiency of the immune response, therefore, specific treatment and vaccination approaches should be developed. The efficiency of phagocytic activity is a significant organism's indicator which decreases with the aging of the immune system. The medications are able to influence phagocytosis, having a blocking or activating effect, are important modulators of immune function. We are developing an *ex vivo* assay indispensable for medication screening in human and *Macaca fascicularis* whole blood. *M.fascicularis* is the well-recognized preclinical model having human-like immune responses. This assay is suitable for routine phagocytosis quantification experiments, including distinguishing between particles adhering to the cell surface and ingested particles. For assay verification several published control drugs were successfully used for blocking and acceleration of phagocytosis. Besides, this test is combined with another activity type such as ROS assay and antibody staining. Ongoing experiments evaluating human and animal phagocytosis indices at different ages will provide statistical standards to be used for medication preclinical and clinical trials. This assay assists to find medications for enhancing the immune response and antigen presentation in the elderly. Moreover, screening of medications,

having impact to phagocytosis and further researches of their mechanisms may bring us closer to the comprehension of fundamental process of immune system aging and to provide a perspective for immune response correction.

P762

Gene Therapy For Adenosine Deaminase Deficiency: Post-marketing Experience and Long-term Outcome

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Autosomal recessive adenosine-deaminase (ADA) deficiency leading to severe combined immunodeficiency (SCID) is a life-threatening immunodeficiency, characterized by lymphopenia in B/T/NK subpopulations, failure to thrive, severe infections and autoimmunity. Allogeneic hematopoietic stem cell transplant (HSCT) and Strimvelis, an ex-vivo retroviral hematopoietic stem cell gene therapy (GT) approved in the EU in 2016, are standard of care treatments for ADA-SCID. Here we describe the post-marketing experience of 19 patients (STRIM cohort) up to 5 years of follow up, the largest cohort of patients worldwide with an approved product based on ex vivo transduced CD34+ cells. We also provide an extended follow-up data on 22 subjects treated in the clinical development/named patient program (CDP+NPP cohort, #NCT00598481) and report two patients treated with mobilized peripheral blood CD34+ cells under hospital exemption (mPB-HE). Age at diagnosis and at GT, CD34+ cell dose and vector copy number (VCN) per genome in the drug product were similar in the CDP+NPP and STRIM cohorts. Nineteen patients initially referred for Strimvelis did not receive treatment due to lack of funding, other treatment choice or ineligibility due to different reasons. At data cut-off (March 2023), all 43 patients were alive, with a median follow-up of 5.5 years (interquartile range (IQR) 3.4-13.0) and 2 year intervention-free survival (IFS) (no need for long-term ERT or rescue HSCT) of 88% (95% CI: 78.7%-98.4%).

Long-term persistence of multilineage gene-corrected cells lead to immune-reconstitution were observed. At the last available observation 16/17 CDP+NPP patients and all STRIM patients, reaching 5 years post GT, discontinued IVIg supplementation and responded to most vaccinations. Response to anti aCD3i and PHA were normal at last follow up in all STRIM and CDP/NPP patients who did not failure GT. Native pathogens' infections had a regular evolution and completely resolved with no sequels. Estimated mixed-effects models showed that higher dose of CD34+ infused affected positively the plateau of CD15+ and CD3+ gene corrected cells. In addition, younger age at GT affected positively the plateau of CD3+ gene corrected T cells as well as lymphocytes, CD3 and CD4+CD45RA+ naïve T cell counts. We also observed a significantly higher plateau of transduced CD15+ and CD3+ cells and better dAXP detoxification in STRIM vs CDP+NPP, but no differences in the lymphocyte or T cell reconstitution.

Most adverse events/reactions were related to disease background, busulfan conditioning or immune-reconstitution. A lymphoid T cell leukemia with retroviral insertion near LMO2 emerged 4.7 years post-GT in a patient from the CDP+NPP group, who is currently in clinical remission 26 months after haploidentical HSCT. In the post-marketing population, the safety profile was in line with the clinical development program and we did not find evidence of clonal proliferation, nor new treatment-related events. Due to the risk of insertional oncogenesis, long-term safety monitoring remains important.

Following disinvestment from the current marketing authorization holder, the license will be returned to Fondazione Telethon who has committed to establish a new model of nonprofit commercialization for ultrarare-diseases, allowing to make this gene therapy still available to eligible patients.

P763

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. In accordance with this trend, gene therapy was used to slow down aging or improve muscle performance. 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 clinical trials. 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. Our results mark a major progress in the detection of AAV-based gene doping providing a valuable screening method. Further analysis on patients’ sera receiving different AAVs by several routes of administration will allow to validate the robustness of the assay.

P764

High systemic dosing of AAV 9 vectors : a relevant rat model to study the impact of the peripheral immune response in the liver

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Recombinant adeno-associated viral vectors (rAAV) are efficient and successful viral vectors for the treatment of monogenic diseases after *in vivo* delivery. However, in several clinical trials using high-doses of vectors, adverse events related to an immune system activation and/or vector toxicity, have been described. This has been noted in particular with AAV9 serotype. Here, we investigated the impact of the immune response in the liver after high-dose systemic injection of rAAV9 in a rat model.

Rats were injected with a high dose of a rAAV9 (1e14vg/kg) expressing the GFP reporter gene (n= 21 rats). IFN-γ lymphocyte secretion against transgene product (GFP) was observed as early as day 7 post-injection, and against AAV9 capsid after one month post injection. Surprisingly, despite a comparable peripheral immune response against the vector in all injected rats, our results show that in only 30% of animals (n=6/21), the GFP protein was no more detected in the liver starting from one month after injection. Thus, we carried out a short term *in situ* hepatic analyses to understand the selective loss of transgene expression in some individuals. At day 7 post-injection (n=6), at the time of a transaminase peak, we have systematically observed a liver inflammation with inter-individual variable characteristics. This variability may explain the consequent persistence or loss of transgene product with likely a balance between hyporesponsive and effector immune responses.

Hence, our study shows that the delivery of high doses of rAAV9 vector in rats results in persisting liver transgene expression in 70% animals, despite a peripheral immune response against the vector. Our rat gene transfer model appears relevant as it seems to reflect the inter-individual differences and the balance between immunity and tolerance described in pre-clinical studies of liver gene transfer.

P765

An *in vitro* human plasmacytoid dendritic cell assay demonstrating CpG level-dependent induction of IFNα to the AAV transgene

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Recombinant Adeno Associated Virus Vectors (AAV vectors) are used as a gene therapy vehicle for the treatment of rare diseases. AAV vectors containing hypomethylated cytosine-phosphate-guanine (CpG) motifs can induce innate and adaptive immune responses. CpG

motifs in AAV vector genomes can activate Toll-like receptor 9 (TLR9), an endosomal innate immune receptor for sensing foreign DNA, mainly expressed by plasmacytoid dendritic cells (pDCs) and B cells in humans. TLR9 signaling initiates expression of proinflammatory cytokines and type I interferons (e.g. IFNα). These innate immune cytokines induce an anti-viral state and enhance antigen presentation, potentiating antigen-specific adaptive immune responses. Reports using mice reveal that sensing of AAV vector CpG motifs via TLR9-MyD88–type I IFN signaling in pDCs is key to induction of adaptive immune responses including cytotoxic T cell responses. Importantly, these experiments also show that the CpG-depleted vectors exhibit prolonged transgene expression and abrogated adaptive immune responses. In Hemophilia B gene therapy clinical trials, use of AAV vectors containing elevated levels of CpGs correlated to the absence of durable transgene expression (<3months), possibly due to enhanced innate signaling via the CpG motifs. Therefore, AAV-directed immune responses may be dampened by reducing the CpGs contained in the AAV vector genome.

We have developed an *in vitro* assay to determine the impact of differential CpG levels in AAV vector genomes on TLR9 activation. Primary human peripheral blood pDCs from a single donor were incubated in the presence of a self-complementary (sc) AAV9 vector containing a transgene modified with different levels of CpGs, or with AAV9 empty capsids. Anti-AAV antibodies were concurrently used to enhance intracellular AAV uptake. After 24 hours, IFNα production was measured from pDCs as a marker of TLR9 activation. We observed dose-dependent induction of IFNα production that correlated to CpG levels in the transgene, albeit over a narrow dynamic range, and that was abrogated upon TLR9 inhibition. IFNα production was not observed in response to empty capsids. Vectors lacking CpGs in the transgene led to IFNα production, suggesting that TLR9 may be stimulated by CpG motifs outside of the transgene. Testing of a single stranded AAVhu37 vector genome also revealed CpG-mediated induction of IFNα from pDCs. Flow cytometric analysis after incubation of scAAV9 vectors containing CpG motifs with pDCs and human peripheral blood naïve T cells from a separate donor confirmed TLR9-mediated activation of pDCs (costimulatory CD40 upregulation) and activation of CD4+ and CD8+ T cells (CD69 upregulation). Together these data indicate that this in vitro pDC assay could potentially be used to screen AAV vectors for CpG-mediated immune activation by utilizing IFNα production from human pDCs as a downstream marker of TLR9 activation.

P766

Early T-cell activation after recombinant Adeno-Associated Virus delivery

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Adeno-associated virus vectors (AAV)-mediated gene therapy is a promising treatment for human monogenic diseases. Despite their high safety profile, clinical trials have shown that their efficacy can be severely impaired by immune responses to AAV products, in particular T cell-mediated immune responses. It has been established that conventional dendritic cells are licensed by plasmacytoid-derived type I interferon to prime specific CD8+ T cells. Toll-like receptor 9 sensing of unmethylated CpG motifs in AAV genomes is core in this process and specific CD8+ T cells appear one week after AAV administration. However, the early events of antigen presentation that occur after AAV injection have been poorly investigated.

To study the early antigen presentation of AAV-derived MHC class I epitopes, we used an AAV vector that either bears the OVA_{257} epitope on the capsid, or encodes for it under the control of an ubiquitous promoter, and followed the activation of OVA_{257}-specific transgenic (OT-I) CD8+ T cells. We showed that AAV vector genomes are detected in the draining lymph nodes as rapidly as 1 hour after intramuscular injection. Moreover, capsid-derived OVA_{257} epitope is also detected in a dose dependent manner by OT-I CD8+ T cells at 24h, suggesting rapid lymphatic drainage of AAV particles and presentation by resident antigen-presenting cells. Regarding the transgene product, a delay of antigen presentation was expected due to AAV genome processing and expression of the OVA_{257} transgene. Surprisingly, the transgene product was also detected in a dose-dependent manner by specific CD8+ T cells as soon as 24 hours post gene transfer. This detection increased thereafter and occurred with multiple AAV serotypes. Transgene-specific CD8+ T cells were shown to proliferate at day 4 in the draining lymph nodes and a significative expansion is observed at day 7, indicating an efficient T cell priming. Finally, transgene presentation was also observed after administration of AAV vectors when transgene expression was modulated by different promoters or by a miR142.3pT de-targeting strategy.

Overall, our study highlights a high level of presentation of both capsid and transgene products few hours after AAV delivery, and indicates that CD8+ T cells are rapidly primed by AAV.

P767

Extreme inter-individual variability of subretinal AAV-induced immune response even in a highly standardized context

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Ocular gene therapies that use adeno-associated viruses (AAVs) to deliver genes have been successfully applied in some inherited retinal diseases. However, inflammation resulting from immune responses to the AAV or the transgene remains an important concern. Anti-transgene and anti-capsid immune responses have been reported in some patients who received AAV gene therapy, which adversely can affect safety and efficiency of the therapy. Immunomonitoring and pertinent biomarker finding is a crucial challenge at present for patient management. Heterogeneity of immune behaviour between patients is traditionally thought to be mainly the consequence of genetic background diversity and previous exposure to different pathogens over the lifetime. We have previously reported in a syngeneic murine model that one subretinal injection of AAV8 systematically induces an anti-transgene T-cell response, but with different levels of intensity. Here, we analysed complementary innate and adaptive immune components to have a better understanding of the immune consequences subsequent to a subretinal AAV injection and the degree of individual diversity in a standardized context.

To reach this aim, transgene cassette encoding the HY male antigen, containing MHC class I- and MHC class II-restricted T-cell epitopes, immunodominant in female H-2b background, was packaged into AAV8 under the ubiquitous PGK promoter. Several experiments were done with a single subretinal injection of high dose AAV8-HY was performed at day 0 in immunocompetent adult female wild type C57BL/6 (H-2b) mice. At day 21, RT-droplet digital PCR (ddPCR), ELISpot, in vivo cytotoxicity and ELISA were done to evaluate the transgene expression, antigen presenting

cell markers, T-cell responses and antibody production. Principle component analysis, correlations, and diversity index have been calculated based on the data from each readout.

Surprisingly, we found that despite a systematic local and systemic immune reactivity following the subretinal injection of high dose AAV8-HY, a very strong variability of the immune response occurs in the different mice of the experiments. Indeed, none of the mice display the same immune response profile while the genetic background, the injected product, the housing conditions, and the experimental procedure where exactly the same.

We provide experimental evidence that an extreme inter-individual variability of subretinal AAV-induced local and systemic immune response profile occurs even in a syngeneic and highly standardized context. This leads to consider that in patients subjected to ocular gene therapy, in addition to variable genetic backgrounds and different immune history over their lifetime, immunomonitoring should be expected as basically very heterogeneous, and one immunological parameter should certainly not be sufficient to predict and follow immune response behaviour.

P768

IgG depletion with imlifidase - a potential way to increase the eligible patient population for AAV based gene therapies

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Pre-existing neutralising antibodies can be a major obstacle for adeno-associated virus (AAV) based gene therapy, as they limit the therapeutic efficacy, increase the risk of adverse events, and reduce the eligible patient population by up to 30-60% depending on AAV serotype. However, these antibodies can be effectively depleted by imlifidase, a highly specific IgG-cleaving cysteine protease originating from *Streptococcus pyogenes*. Within a few hours after dosing, imlifidase efficiently cleaves more than 99% of all IgG, creating an approximately one-week antibody-free window, before newly synthesised IgG rebounds. IgG levels are normally restored to their initial levels after 2-6 months. Imlifidase obtained a conditional marketing authorisation as Idefirix™ on 25 August 2020 in Europe* for desensitisation of highly sensitised adult kidney transplant patients, and there are several ongoing clinical trials in a variety of indications, with IgG antibodies and Fc-mediated effector responses as their common denominator. Clinical trials for imlifidase in kidney transplantation, anti-glomerular basement membrane disease and Guillain-Barré syndrome demonstrated acceptable safety profiles, with the most common adverse events being infection (16.5%) and infusion-related reactions (5.6%), resolving within 90 min. The most common treatment-related serious adverse events reported in this population were pneumonia (6%) and sepsis (4%). There were no serious adverse events reported in any of the 35 healthy subjects treated with imlifidase. All in all, antibody depletion with imlifidase could be a solution for patients excluded from participation in gene therapy clinical trials because of pre-formed immunogenicity towards the AAV vector. The therapeutic potential of imlifidase as pre-treatment to gene therapy is currently being evaluated for AAV based gene therapies in collaboration with Sarepta Therapeutics, Genethon and AskBio.

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P769

Identification of B-cell receptor sequences against Adeno-Associated Virus (AAV)8 empty capsids from whole blood of healthy clinical trial participants

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Recent studies highlight the ability to isolate anti-AAV antibody-encoding B-cell receptor (BCR) sequences from naturally– and clinically-exposed humans, and that these antibodies can be cross-reactive to multiple AAV serotypes. While a plethora of data on non-human anti-AAV antibodies exist, it is unclear how healthy human antibody responses develop *de novo* after exposure to clinically-relevant dosages, how this might affect epitope targeting, or how isotype-usage evolves. Herein, healthy participants were administered 2E12 AAV8 empty capsid particles/kilogram, and whole blood RNAseq was performed longitudinally to Day 90 (D90), as well as immunological measurements like neutralizing antibody (NAb) titering. Bioinformatic approaches deconvoluted the BCR repertoire, and antibody cross-reactivity and isotype were monitored via custom MesoScale Detection (MSD) assays. By D8, 7 of 8 participants exhibited NAb cut-points of >1:100, with all participants ≥1:75 at D90 and serotype cross-reactive. Fresh blood flow cytometry indicated >5-fold changes in antibody-secreting cells at D8 that was recapitulated by CiberMed transcriptomic deconvolution. Repertoire analyses detected complementarity-determining region (CDR)3 elongation and contraction over time, and expansion of BCR clonotypes with moderately conserved gene usage compared with other studies. Immunoglobulin (Ig) M, A, and G clonotypes were observed, with notable amounts of IgA gene usage, and protein expression corroborated by MSD. In some participants, BCRs were detectable prior to AAV8 administration, indicating they were “pseudo” negative to AAV8 – NAb titers below cut-off criteria but possessing cross-reactive antibodies detectable by MSD. Together, these data aid our understanding of human anti-AAV antibody responses and may allow for creation of next-generation AAV-detecting approaches.

P770

Development of Adeno-associated virus (AAV5) variants capable of reducing the immunogenicity by targeting antigenic residues on the capsid surface

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Numerous clinical trials and approved gene therapy drugs demonstrated AAV as a suitable delivery vector for gene transfer in various monogenic diseases. One of the hurdles facing rAAV

vector administration is the pre-existing humoral immunity against wtAAV capsid, resulting in an inefficient gene expression and an exclusion of patients from treatment.
This project aims to develop AAV5 variants capable of evading pre-existing neutralizing antibodies (Nab) by targeting potential antigenic residues on its capsid with amino acid substitution to alanine. Mutated variants were tested for their production and transduction efficiencies *in vitro*. Nab titer referred by the percentage of transduction efficiency on HEK293 cells was determined for each variant and compared to wtAAV5, using a cell-based assay in presence of Nab in human (n=12) and porcine (n=8) serum samples as well as pooled intravenous immunoglobulin (IVIg). Human and porcine serum showed varying Nab titers against wtAAV5.
We managed to generate AAV5 variants carrying single point mutation (n=40) and combined point mutations (n=8). 17 variants were able to escape Nab in human sera, characterized by reducing Nab titer compared to wtAAV5. Testing these variants against Nab in pig serum samples proved that 7 variants were able to escape Nab as in human serum. Visualizing models of AAV5 variants carrying point mutations illustrate the location of antigenic resides on the outside virus capsid's surface.
Taken together, we have identified AAV5 variants with reduced neutralization by Nabs. Further studies are necessary to investigate whether transduction efficiency is preserved in clinically relevant animal models.

P771

Polyfunctional T cells and unique cytokine clusters imprint the anti AAV2/AAV9 vector immune response

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Polyfunctional T cells (PfTcs) programmed to perform activities such as degranulation of lytic enzymes and simultaneous production of multiple cytokines are associated with more effective control of viral infections. Immune responses to adeno-associated virus (AAV) vector delivery systems are critically influencing therapeutic efficacy and safety of gene therapy. However, knowledge on PfTcs in anti-AAV immune responses is scarce. Here, we investigate this knowledge gap by exploring the polyfunctionality of primary human T cells of healthy donors after *in-vitro* exposure to AAV2 or AAV9 vectors.

By performing proliferation assays of co-cultured T cells and AAV pulsed monocyte-derived dendritic cells from healthy donors we demonstrated a T cell reactivity of 43% or 53% to AAV2 and AAV9 vectors, respectively. We could corroborate this frequency in a second screen using another set of healthy donors measuring CD25 / CD71 T cell activation.

Single T cell secretome analysis of reactive donors uncovered a T_h1 pro-inflammatory, cytolytic and chemoattractive cytokine release after stimulation with AAV2 or AAV9 vectors. 12.4% and 9.6% of these stimulated T cells displayed a polyfunctional cytokine response, respectively, including elevated polyfunctional inflammation indices. These responses were characterized by cytokine clusters such as GrzB, MIP1-α and TNF-α released in combination by single T cells.

Overall, our PfTc insights into immunological dynamics associated with AAV vector serotypes provide valuable information for advancing gene therapy safety, vector selection, immunogenicity assessment and better patient selection for AAV gene therapy.

P772

Orthogonal Approach for AAV Immunogenicity Assessment: Evaluating Total and Neutralizing Antibodies

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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. Among them, the presence of antibodies against AAV has been shown to affect the outcome of *in vivo* AAV-mediated gene therapy. The most direct impact occurs during administration through capsid neutralization, or by triggering immune reactions such as activation of the complement system.

Currently, it is recommended by different industry leaders to analyze both the presence of total binding antibodies (TAbs) as well as neutralizing antibodies (NAbs). Here we present Svar's AAV immunogenicity solutions that allow for assessment of both TAbs and NAbs in two highly customizable assays. First, TAbs are assessed with a standardized immunoassay with a high sensitivity, allowing the evaluation of the presence of TAbs before or after administration of AAV vectors. In a second assay, NAbs are assessed using a novel cell-based *iLite*® AAV platform to avoid the Nab-mediated reduction of AAV transduction. The *iLite*® AAV Platform is composed of a new two-component system and can be used for detecting and optimally quantitating anti-AAV NAbs directed against the capsid in a test sample.

These two platforms combine the advantages of both immuno- and cell-based assays. By offering reliable and customizable solutions for assessment and differentiation of humoral antibody responses against AAV vectors we respond to 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.

P773

Improvement of a neutralizing antibody assay for 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. When using HEK293 and Huh-7 cells, commonly used firefly luciferase assays required more than 100,000 vector genomes (vg)/cell for AAV5 and 10,000 vg/cell for AAV8 or AAV9 to confirm sufficient gene expression. To enhance the transduction efficiency of AAV, we tested to add a wide variety of compounds to the cell culture medium and found that the saccharides enhanced transgene expression efficiently regardless of AAV serotype. Additionally, by the combination of NanoLuc, a highly sensitive luminescent luciferase, as a reporter gene, the amount of AAV vector required for the NAb assay was greatly reduced to 100-1,000 vg/cell for AAV5 and 10-100 vg/cell for AAV8 and AAV9. We confirmed that this assay is significantly sensitive for NAb against AAV5, AAV8, and AAV9 compared to an assay using firefly luciferase. The sensitivity of NAb was almost equal using either HEK293 or Huh-7 cells and each cell can be selected according to the infectivity of AAV serotypes in this assay. In conclusion, we established an assay that can detect NAb against diverse AAV serotypes with high sensitivity using commonly available cell lines.

P774

Immunomonitoring requirements in AAV based gene therapy clinical trials: where do we stand?

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Recombinant adeno-associated viruses (rAAV) are being largely used *in vivo* as viral vectors in gene therapy clinical trials. Promising results have been obtained with several rAAV products as illustrated by six drugs currently approved (Luxturna, Zolgensma, Roctavian, Hemgenix, Upstaza and Elevidys) in Europe and USA. However, host immune responses to the AAV capsid and/or the transgene product, remain one of the major concerns for the safety and efficacy of rAAV-based strategies. Immune responses including anti-AAV neutralizing antibodies, T cell-mediated cytoxicity, and more recently, complement activation, have been described in patients after systemic rAAV-based gene transfer. Consequently, immune monitoring has become mandatory during regulatory preclinical and clinical studies as it is required by regulatory agencies. A large number of immune assays and approaches have been developed over the past years, by academic and private research laboratories, to support *in vivo* rAAV-based gene therapy studies. However, validation and standardization of these methods are still challenging because of their technical features and the limited characterized specific reagents available.

Here, we highlight the major steps required towards the development, validation and use of immune monitoring assays to robustly support patient follow-up in clinical trials using rAAV products. From the selection of the appropriate assays to the standard operating procedure, we will discuss how to prepare, validate, define the acceptance criteria and document the immunoassays to be in line with the regulatory agencies' requirements. We will also give an insight on the major hurdles that could be encountered during clinical immune monitoring. In addition to the absence of standardized materials and methods, many other challenges remain such as patient sample availability especially for children affected with genetic diseases or the complexity of the

interpretation of immunological results in correlation with other clinical parameters. Finally, we will describe the general work-flow to implement clinical immune monitoring, in line with Good Clinical Laboratory Practices (GCLP) and other regulatory guidelines. Beyond the method validation itself, other steps, such as definition of responsibilities, personnel training or data and material traceability have to be implemented and controlled by the immunology laboratory to assess safety and efficacy endpoints, while ensuring the quality and integrity of the data.

P775

Exploring the relationship between senescence and purine metabolism: new therapeutic possibilities

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One of the main challenges in biomedical sciences focuses on discerning the molecular mechanisms that cause aging, which makes it a hot topic. Among the many hallmarks of aging, it presents an arrest of proliferation. This aspect is defining of the senescent state also characterized by a pro-inflammatory secretome. Senescence-associated secretory phenotype or SASP have the capacity of paracrine senescence transmission. During aging the number of senescent cells increase triggering an inflammatory microenvironment, leading to the develop of age-related diseases. Purine metabolism has an important role in the formation of *de novo* purines which are part of the ribonucleic acids. Several proteins participate in the purine metabolism like the metalloprotease CD13 that transform peptides in aminoacids, which induce the reaction cascade of the purine formation. Before cell division, the DNA duplicates incorporating these purines. The aim of this project was to determinate the role of CD13 in senescence process. For that, we treated mesenchymal stem cells from umbilical cord stroma, chondrocytes (TC28a2 line) and epithelial ovarian cancer cells (OVCA SK-VO-3 line) with CD13 inhibitor bestatine, for induce senescence due to the lack of purine formation and subsequent incorporation in the DNA. We evaluated senescence by proliferation assays and presence of senescence (p21) and SASP (IL-6 and IL-8) biomarkers. The results show proliferation decrease and biomarkers increase respect the control: gene expression (*IL8*, *IL6*, *CDKN2A*, *CDKN1A*); protein expression (p21); β -galactosidase activity . This study highlights the purine metabolism dysregulation involved in the senescence establishment process, positioning CD13 as a potential target in the development of senodrugs treatments against age-related diseases.

P776

Extracorporal Photopheresis (ECP) induces apoptosis and inhibits T cell proliferation

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Extracorporal Photopheresis (ECP) is an immunomodulatory strategy that is based on the induction of apoptosis of subsets of patient's mononuclear cell (MNC) enriched leukocytes with the aim to positively influence the micromillieu that entertain inflammatory conditions. During ECP, the apheresed cells first undergo the incubation with 8-Methoxy Psoralen (8-MOP) followed by exposure to ultraviolet (UV) irradiation. 8-MOP, a naturally occurring inert compound, forms covalent bonds with DNA, proteins, and cell membranes upon UVA irradiation. This triggers selective apoptosis and other forms of (programmed) cell death. A resulting net effect includes subsequent modulation of dendritic cells and immune tolerance.

Here we present in-vitro data regarding the influence of ECP treatment on apoptosis of peripheral blood (PB) MNCs and on the proliferation of T lymphocytes of three healthy donors as a proof of concept. The leukocyte concentrates used for this purpose were diluted with 0,9% NaCl and treated with 8-MOP +/- ECP. After isolation of PBMNCs from ECP-treated and ECP-non-treated leukocyte concentrates, 7 to 11x10^8 cells were incubated for 48 hours before apoptosis and proliferation assays were performed. Apoptosis of the PBMNCs was determined by staining the cells with annexin-5 and 7-AAD and flow-cytometric quantification. Our data show that ECP significantly decreases the viability of PBMNCs. While the viability of non-ECP treated samples was 77-86%, the ECP-treated samples showed viabilities ranging between 37% and 65%. Thus, 48 hours after ECP treatment, 25% - 56% of cells exhibited apoptosis. To analyze the proliferation capacity of T lymphocytes, PBMNCs from 3 healthy donors were stained with Violet Proliferation Dye 450 (VPD450) and T cell receptor stimulated with anti-CD3/CD28 mediated cross-linking. VPD450 is a non-fluorescent dye, which diffuses passively through the cell membrane, after which it becomes highly fluorescent in viable cells by esterase cleavage. After cell division, fluorescence intensity per cell is reduced based on its dilution into daughter cells. Fluorescence intensity quenching is thus a measure of active cell division. After stimulation for 72 hours as mentioned above, PBMNCs were stained with anti-CD3, anti CD45 and 7-AAD to separate T cells from other PBMCs. T cell proliferation was analysed by flow cytometry. Proliferation capacity of the T lymphocytes after ECP treatment decreased significantly to 0%, 0.2%, and 32.4% as compared to 90%, 89% and 91% without ECP treatment, thus confirming the efficacy of ECP.

In view of a surprising shortage of ex-vivo data, in a pilot set of experiments ECP treatment induces apoptosis and inhibited the proliferative capacity of T lymphocytes indicating potential modes of its overall immunomodulatory properties. The effects (humoral and cellular) of the ECP need to be investigated more systematically. However, it appears that this method could be implemented in several inflammatory conditions by controlling immune responses and inducing an anti-inflammatory environment. More studies need to address this possible expansion beyond its use in chronic graft vs. host disease and in primary cutaneous lymphoma. We will for example investigate the role of ECP in inflammatory complications of cellular therapies such as cytokine release syndrome.

P777

Simple, rapid and robust bioluminescent cell-based assay for detecting neutralizing antibodies against AAV in serum.

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Adeno-associated viruses (AAV) are widely used vectors for gene therapy, capable of delivering transgenes as treatment for monogenic diseases. Individuals with pre-existing immunity to AAVs are less likely to benefit from AAV-based therapies due to both, a reduction in cellular AAV uptake and activation of a cytotoxic T-cell response to transduced cells, decreasing efficacy or changing the safety profile. A robust and reliable neutralizing antibody (NAb) assay is necessary to determine eligibility for treatment as well as to identify qualified individuals for clinical trials. Here we present a cell-based assay using NanoLuc® luciferase to detect NAbs in serum samples. This NanoLuc® AAV reporter system displays several advantages over existing assays such as: superior sensitivity, very low MOI required (100 – 3000 depending on serotypes), short 24h assay time, and detection using a standard luminometer. We tested 60 human serum samples for the presence of NAbs against a panel of AAV serotypes 1—10 and engineered variant AAV-DJ. We found that a large fraction of the population had pre-existing NAbs to at least one serotype, and of the pre-exposed individuals a majority displayed NAbs against multiple AAV serotypes. Interestingly, fewer samples were seropositive for AAV-DJ than for either parental serotype (AAV2, AAV8 or AAV9), highlighting the benefit of capsid engineering. Furthermore, a broad dynamic range of our assay enables categorizing the serum samples into 4 groups: negative, low, medium and high NAb tiers. Representative samples of each group were assayed for neutralizing titers (ND_{50}). The ND_{50} values of the samples showed agreement with their assigned groups. To demonstrate the robustness of our assay, we conducted a mouse study with 40 mice divided into 4 groups and administered AAV9 at varying doses. This study showed a strong correlation of NAb levels to the injected AAV viral loads. Taken together, our assay is rapid, highly sensitive and reliable; the NanoLuc® AAV reporter technology enables precise measurement of NAbs against AAVs in serum. Further validation of the assay is required for future use in a clinical setting.

P778

Human Chimeric AAV Antibodies – Advanced Standard for Serological Assays

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Recombinant adeno-associated virus (AAV) vectors have become leading tools for viral gene therapy. Alongside the many advantages, like its apathogenic nature, there are some concerns about the safety and efficiency of AAV-based gene delivery into human cells. Depending on the AAV serotype, many humans in the general population have developed antibodies against AAV as a result of naturally acquired infections. Thus, serum antibodies against many of the wild-type AAV capsid proteins can be found in a significant proportion of the population. Intriguingly, pre-existing immunity to AAV might affect efficiency and safety of the gene transfer using AAV vectors. While the use of a high dose of AAV particles might lead to unwanted immune reactions that might put the patient at risk, the use of a low dose might fail to elicit the desired therapeutic effect. Finding the right dose without causing severe side effects but ensuring efficiency of the therapy still is a major challenge in every therapeutic approach. It is an even greater challenge in the area of virus-based gene therapy due to the limitation of a single vector application to each patient. Therefore, testing for pre-existing AAV antibodies in patient sera remains an indispensable step for the inclusion of patients into gene therapy programs.

To develop reliable and reproducible AAV serology assays for the evaluation of patient groups, positive controls are crucial. For this purpose, well-characterized sero-positive samples or purified

human antibodies that are verified to bind or even neutralize the corresponding AAV serotype are valuable controls.

To provide this suitable and fully characterized positive control for serological analysis of patient sera, we developed recombinant humanized chimeric AAV antibodies against a number of different AAV serotypes, including AAV1, 2, 3, 5, 6, 7, 8, 9, rh10, and rh74. Our new recombinant humanized chimeric AAV antibodies have been developed based on our exclusive portfolio of neutralizing anti-AAV mouse monoclonal antibodies. The antibodies have identical sequences to our conventional mouse AAV antibodies (e.g. ADK1a, A20, ADK5b, ADK8, ADK9), but contain a humanized Fc region to allow comparability with antibodies from patient sera. Thus, these antibodies can be detected in AAV serology assays based on an anti-human secondary antibody system.

Here, we show characterization data of our new humanized chimeric AAV antibodies, including cross-reactivity, stability and neutralization. There is high similarity regarding cross-reactivity when compared with our conventional mouse monoclonal parental clones. The applications of these antibodies include dot blot analyses, neutralization assays as well as ELISA.

P779

Novel method for quantifying Anti-AAV Neutralizing Antibodies using AVV producing *iLite*® cell-based assay

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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 be used to address key parameters for regulatory acceptance including vector potency or determination of neutralizing antibodies (NAbs).

Here we present a novel two-component system for the detection and quantification of NAbs directed against recombinant AAV vectors, the *iLite*® AAV Platform. This platform can be used for detecting and optimally quantitating anti-AAV NAbs directed against the capsid in a test sample. The availability of reporter cells in a frozen, thaw & use format, obliviates the need for cell culture or specialized equipment and provides a means for obtaining highly reproducible results superior to those obtained using the same cells maintained in culture. Furthermore, the time necessary to run the assay is half of the time required in the current alternatives.

These features combined allow the advantages of a cell-based assay to quantify neutralizing anti-AAV antibodies, as recommended by regulatory agencies, to be combined with the versatility and ease of an immune-detection assay for use in AAV-based gene therapy trials.

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Investigate AAV Vector Immunogenicity in humans: mechanistic understanding to drive successful gene transfer

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Gene therapy using vectors based on adeno-associated viruses (AAV) has emerged to treat debilitating diseases such as metabolic and neuro-muscular genetic disorders. In fact, the recent approvals of Luxturna, an AAV2 vector for the treatment of Leber's congenital amaurosis, and Zolgensma, an AAV9 vector for the treatment of spinal muscular atrophy, have boosted their potential to treat a wider range of diseases. With the increased use of recombinant AAV (rAAV) for gene therapies, we have started to deeply investigate their immunogenicity risks to better understand the innate and adaptive immune responses triggered upon exposure to rAAVs. Although wild type AAVs can infect humans, they are not causing any disease, but can induce a mild humoral and/or cellular immunogenic responses. In fact, the broad application of rAAV-based-gene therapy is limited by the pre-existing immunity induced by the natural exposure to wild type AAVs. The activation of T cells can lead to rejection of transduced cells, while B cells can produce AAV neutralizing antibodies, which may limit or prevent a successful transduction of target cells. The seroprevalence of pre-existing immunity to viral vectors varies across capsid serotype, age, gender, and geographical location. The pre-existing antibodies can directly bind the capsid preventing the binding and internalization of the vectors, thus inhibiting transduction of target cells and the therapeutic activity. Additionally, the cellular and humoral immune responses are also possible against the expressed transgene protein, thus preventing the therapeutic efficacy.

In parallel to the pre-existing immunity, also the *de novo* host immune responses (innate and adaptive) can determine the safety and effective treatment. Several immunogenicity mitigation strategies are under investigation to avoid the immune rejection to AAV vector or allow patients with pre-existing antibodies to have access to the treatment and enter the clinical studies. Finally, we believe that our work and efforts can contribute to a comprehensive understanding of the mechanisms driving AAV vector immunogenicity in humans, with the final aim of developing safe and effective therapies.

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Bioorthogonal conjugate platform development for in vivo gene editing and therapy

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Bioconjugation of proteins can substantially expand the opportunities in biopharmaceutical development, however, applications have been limited for the gene editing machinery despite its tremendous therapeutic potential. Here, we introduce a self-delivered nanomedicine platform based on bioorthogonal CRISPR/Cas9 conjugates, which can be armed with a chemotherapeutic drug for combinatorial therapy, or functionalized with a targeting antibody for specific delivery.

We sought to demonstrate that the nanomedicine platform can be applied for combinatorial therapy by incorporating the anti-cancer drug olaparib and a carrier polymer, or cell-specific delivery by conjugation with a HER2-targeted antibody, and evaluated in inducing anti-tumor effects in mouse cancer models of the breast and ovaries.

In brief, we developed a bioorthogonal Cas9 platform by incorporating unnatural amino acids to the Cas9 protein. We explored different sites for incorporation and selected the most suitable variant for further experiments. We demonstrated the successful conjugation of the Cas9 variant with chemotherapeutic drug olaparib and a carrier polymer, forming self-condensed nanocomplexes. The efficacy of bioorthogonal Cas9 conjugate nanocomplexes for in vitro gene editing and combination therapy was evaluated, demonstrating the synthetic lethality effect by gene editing of RAD52 and PARP inhibition by olaparib, that target the DNA repair pathway. Furthermore, *in vivo* delivery of the bioorthogonal Cas9 conjugate nanocomplexes led to significant gene editing and synergistic effect on suppression of tumor growth in a BRCA-mutant cancer model.

We also demonstrated that cell-specific delivery could be achieved by functionalization of the bioorthogonal Cas9 variant with a HER2-targeting antibody. The bioorthogonal Cas9-antibody conjugates showed greatly enhanced delivery and gene editing of the PLK-1 gene in HER2-positive ovarian cancer cells. Furthermore, *in vivo* delivery of the bioorthogonal Cas9-antibody conjugates was able to majorly suppress tumor growth, by controlling cell cycle progression in the tumor.

In sum, the bioorthogonal Cas9 conjugate platform could be efficiently delivered, induce gene editing, and co-deliver chemotherapeutic agents or enable cell-specific delivery in tumors. The study will bring major impact to the field by providing a robust and safe gene therapy platform, that can induce synergistic effects and minimize side effects *in vivo*. We anticipate that this versatile nanomedicine platform could be extended to other diseases, showcasing its potential for revolutionizing biopharmaceutical development.

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TraffikGene: a versatile platform for nucleic acid delivery based on amphiphilic peptide carriers

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Despite the vast potential that nucleic acid therapeutics (NATs) hold to address the genetic basis of a wide variety of diseases, relatively few effective RNA-based treatments have been successfully developed in large part due to the “delivery problem.” Systemically administered naked RNA is quickly degraded by endonucleases and can provoke adverse immune reactions. It has no affinity for target cells, and its off-target actions can be toxic. To protect and transport RNA, viral vectors, lipid nanoparticles (LNPs) and polymers have been tried in recent years. However, all these systems suffer from one or more limitations such as: toxicity due to carrier immunogenicity, optimization challenges, complex formulation or manufacture, and it is still hard to achieve accurate targeting to tissues of interest. All of these unsolved challenges continue to limit the ability to fully capture the potential of RNA therapeutics, which have the power to revolutionize medicine.

*TraffikGene´*s delivery platform allows for the development of simple to formulate and manufacture vehicles, that are safe and non-immunogenic, to carry RNAs to the cytosol of different cells and tissues. By using short, reactive cationic peptides, it is possible to attach selected hydrophobic tails onto the peptide scaffold to yield biodegradable amphiphilic peptide carriers. The straightforward modularity of peptide and tail components allows for high-throughput generation and screening of libraries of hundreds of delivery candidates. Carrier attributes (cargo preferences, delivery efficiency, distribution, etc.) can be chemically refined by means of structure-activity analysis to accelerate lead discovery on-demand. This unique platform approach has the potential to enormously facilitate and de-risk drug development processes for NATs and further provides an additional layer of strong IP.

These single component vehicles can easily complex with genetic cargos to yield nanoparticles for in vivo delivery of mRNA, siRNA, pDNA or ribonucleoproteins such as Cas9. Due to the unique chemical properties of TraffikGene's carriers, after they reach the cell's interior, the nanoparticles are disassembled to promote endosomal escape, yielding innocuous metabolites which are processed by cells, leading to non-toxic intracellular delivery.

The functional versatility of this technology has been demonstrated in a variety of in vivo assays. Formulations of an mRNA encoding for a specific antigen with TraffikGene's carriers, resulted in strong and durable immune response against those antigens. These results open new possibilities to employ this delivery technology in the development of novel NATs in fields like cancer vaccination or rare diseases, both areas that we are investigating.

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Revolutionizing neovascular AMD treatment: Electrotransfection-based dual-gene therapy platform

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Ocular gene therapy has entered clinical practice. Although viral vectors are currently the best option for replacing and/or correcting genes, the optimal method for delivering these therapies to retinal pigment epithelial (RPE) and/or photoreceptor cells still needs to be improved to increase transduction efficiency and reduce iatrogenic risks. Beyond viral gene replacement therapies, non-viral gene delivery approaches offer the promise of fine, sustained expression of secreted therapeutic proteins that can be tailored to the evolving stage of the disease and can treat more common retinal diseases, such as age-related macular degeneration (AMD). We have developed a cutting-edge minimally invasive delivery platform based on the principle of electroporation, using a proprietary electrotransfection injection system to deliver plasmids to the ciliary muscle, enabling sustained expression of therapeutic proteins in the eye. This technology overcomes the significant limitations associated with more invasive gene therapy options requiring subretinal injections and expands the diversity of therapeutic proteins that can be delivered to the eye, as DNA plasmids have virtually no cargo size limit. Here, we present the rationale and design of EYS809, a first-in-class treatment for neovascular (wet) AMD, intended as an alternative to repeated anti-VEGF intravitreal (IVT) injections, which have been associated with burdensome treatment and poor long-term visual outcomes. EYS809 is a dual-gene plasmid encoding two potent therapeutic proteins: aflibercept, a VEGF trap protein with antiangiogenic effects, and decorin, an intrinsic leucin-rich proteoglycan which plays a key role in inhibiting angiogenic factors and regulating various pathways involved in retinal disease, including choroidal neovascularization (CNV), RPE barrier disruption and epithelial-mesenchymal transition (EMT) of RPE cells, which

contribute to subretinal fibrosis in wet AMD. In a rat model of persistent CNV, EYS809 outperformed intravitreal aflibercept, demonstrating a continuous reduction in vascular leakage. In addition, EYS809 significantly promoted the restoration of the RPE monolayer over the CNV lesion, indicative of RPE wound healing. Furthermore, in the neuroretina, EYS809 downregulated the expression of extracellular matrix-related genes (*MMP2*, *COL1A1*, *and COL4A1*) involved in subretinal fibrosis. Collectively, these findings underscore the potential for EYS809 to promote sustained disease regression and diminish the need for anti-VEGF injections over the long term.

P784

Intein-mediated *cis*- and *trans*-splicing enables tighter control of *Sleeping Beauty* transposase activity

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DNA transposon systems such as *Sleeping Beauty* (SB) are useful nonviral vectors mediating efficient and long-term transgene expression. In SB transposition, stable genomic integration of a gene of interest from a DNA donor molecule is obtained through a “cut-and-paste” reaction catalysed by the SB transposase. Conditional activity of the transposase can be achieved by using tissue-specific or drug-inducible promoters and can be an advantageous feature for more precise genome engineering and safer gene therapies. Leaky expression from these promoters, however, can lead to undesired and irreversible effects, as very little amounts of transposase can be sufficient for the generation of stable integrations. Here we report the use of intein-mediated protein splicing as a way to provide additional control over SB transposition. Based on secondary structures of the functional domains of the hyperactive SB100X transposase, we introduced both *cis*- or *trans*-splicing inteins at specific amino acid positions. We demonstrate that *Npu* DnaE split inteins enable efficient *trans*-splicing of the SB transposase yielding a reconstituted transposase that preserves highly efficient transposition activity. We combined this system with either inducible (Tet-On) or hepatocyte-specific promoters for conditional expression of each of the intein-tagged transposase domains. In addition, a ligand-dependent intein that undergoes *cis*-splicing only in the presence of 4-hydroxytamoxifen (4HT) provided tight post-translational control of the transposase activity with very low background in the absence of 4HT. These novel SB transposase variants permit tighter regulation of SB-mediated genome engineering and hold promise for advancing the field of gene therapy towards more targeted and controlled applications.

P785

In vitro modelling of the Finnish founder mutation responsible for argininosuccinate lyase deficiency and development of a lipid-nanoparticle-delivered base editor system for gene correction in patient primary fibroblasts

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Argininosuccinic lyase deficiency (ASLD) is a urea cycle disorder that results from mutations in the gene *ASL*, which encodes the argininosuccinic lyase (ASL). ASL catalyzes the breakdown of the toxic metabolite argininosuccinate (ASA), and it is strongly expressed in the liver and kidney. Neonatal hyperammonemia characterizes the severe form of the disease, and without treatment, the newborn is at risk of neurological injury and even death. To this day, there is no curative therapy for this disease. Palliative care relies on a strict low-protein diet, nitrogen-scavenging treatments, and in severe cases, hemodialysis and liver transplantation.

We generated corrected and not-corrected isogenic hiPSC lines from skin biopsies of two homozygous patients for the Finnish founder variant *ASL* c.1153C>T. We then compared the metabolic profile of hepatocytes derived from these hiPSC lines and saw a significant decrease in ASA levels within the cells and in the media, 1000 and 100-fold respectively, after gene correction.

As a proof-of-concept for curative gene-editing therapy, we encapsulated our mRNA construct encoding the Adenine Base Editor (ABE8e) and the sgRNA into Lipid Nanoparticles (LNPs), creating a system compatible with in vivo delivery. We assessed the gene-editing efficiency in patient primary fibroblasts employing three different FDA-approved LNP formulations and analyzed the metabolic phenotype of the genetically corrected cells. The gene-editing efficiency was dose-dependent, surpassing 90% even at low LNP doses. Toxicity analysis did not show significant changes in fibroblast death or growth. Finally, we measured the ASA levels in patient primary fibroblasts after LNP treatment and observed a marked significant 10-fold decrease in ASA levels intracellularly and in the media.

P787

Engineered ARRDC1-mediated microvesicles (ARMMs) as vehicles for delivery of genome editing payloads for immune modulation therapeutics

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A growing number of genome editing tools and derivatives continue to expand the range of potential therapeutic applications. However, a large majority of these applications are hindered by the inability to deliver genome editors or derivatives to disease-affected cell types. The most significant challenge is identifying non-viral vehicles that permit safe, transient, and highly efficient functional delivery. Here we report efficient loading of ARMMs with Cas9, ABE (Adenine base editor), CBE (Cytidine base editors) complexed with gRNA (guide RNA) as fully functional ribonucleoproteins (RNP) in ARMMs. We further evaluate functional delivery of these payloads *in vitro* in multiple cell types, including in post-mitotic cells. Furthermore, we provide evidence demonstrating high levels of *in vivo* genome editing, exceeding 60% in some instances. Our data show robust editing of druggable or undruggable immune modulation targets NLRP3 or IRF5, respectively, and in both alveolar macrophages and Kupffer cells. Additionally, in both instances we demonstrate significant blunting of downstream pro-inflammatory cues. In totality our data suggest that ARMMs are promising non-viral vehicles for treatment of diseases of inflammation and fibrosis such as alcoholic steatohepatitis (ASH), non-alcoholic steatohepatitis (NASH), or idiopathic pulmonary fibrosis (IPF).

P788

Virus-like particle mediated protein delivery during ex vivo rat lung perfusion

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Lung transplantation (LTx) is the ultimate therapy for patients with end-stage lung diseases including COPD, pulmonary fibrosis and cystic fibrosis. However, the field of LTx is challenged by a shortage of suitable donor lung and a poor survival of only 59% after 5 years. To expand the donor pool, ex-vivo lung perfusion (EVLP) was clinically implemented to safely increase the usage of donor lungs. EVLP is an innovative technology where donor lungs are ventilated and perfused on a platform so that metabolism can be maintained outside of the body prior to transplantation. Targeting the lung with gene therapy is challenging but EVLP provides a unique window to selectively administer agents that can target the immune response which is responsible for rejection and shorter survival after LTx. CRISPR-mediated genome editing technologies provide unique tools to develop genetic therapies with unprecedented precision and efficacy. However,

since the EVLP time window is limited (6hrs), delivery of editing machinery should be fast and preferably also transient. Virus-like particles (VLPs) are candidate delivery vehicles since they deliver Cas9/sgRNA ribonucleoprotein complexes at high efficiency and increased safety due to their 'hit and go' short presence within the target cell. We hypothesized that VLP-mediated protein delivery in the EVLP system has a high potential for pulmonary gene therapy. To evaluate VLP-mediated protein delivery and uptake in target pulmonary endothelial cells, we designed and produced reporter VLPs that carry either the firefly luciferase (fLuc) or β-galactosidase (βGal) reporter proteins (VLP-fLuc and VLP-βGal respectively). Successful delivery and activity of both the fLuc-and βGal reporter proteins was first demonstrated in HEK293T cells, with the in vitro ONE-GloTM assay and in vitro X-gal staining. Next, transduction was examined and corroborated in primary microvascular endothelial cells. These in-vitro results highlight the potential of VLPs to target the pulmonary endothelium. Finally, we assessed functionality of the fLuc-VLPs in a rat EVLP model. Two independent rat EVLP experiments were performed. After reaching stable EVLP (~1h) either VLP-fLuc or VLP-βGal was administered to the vasculature of the lung and recirculated on the EVLP device for 110 min. Next, 1 ml D-luciferin (50mg/L) was administrated and photon flux generated by fLuc activity was quantified using bioluminescence imaging (BLI). BLI in the control experiment with VLP-βGal resulted in a total photon flux of 5.9x105 (anterior) and 3.0x105 (posterior) photons per second (p/s), whereas the VLP-fLuc treated rat lung resulted in a total photon flux of 3.1x106 (anterior) and 2.2x106 (posterior) p/s. Further histological analysis of β-Gal activity on tissue sections of VLP-βGal treated rat lungs will be carried out to visualize and confirm efficient targeting of the pulmonary endothelium. Taken together, our data show, to our knowledge, for first time, the potential of VLP-mediated protein delivery during rat EVLP. By further advancing VLP-mediated delivery of Cas9/sgRNA complexes in the EVLP setting, we aim to develop a unique strategy to overcome the challenges of rejection after LTx and to find treatments for other pulmonary disorders.

P789

Induction of Bleb Structures in Lipid Nanoparticle Formulations of mRNA Leads to Improved Transfection Potency

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The transfection potency of lipid nanoparticle (LNP) mRNA systems is critically dependent on the ionizable cationic lipid component. LNP mRNA systems composed of optimized ionizable lipids often display distinctive mRNA rich "bleb" structures. Here we show that such structures can also be induced for LNP containing nominally less active ionizable lipids by formulating in the presence of high concentrations of pH 4 buffers such as sodium citrate, leading to improved transfection potencies both in vitro and in vivo. Induction of bleb structure and improved potency is dependent on the type of pH 4 buffer employed, with LNP mRNA systems prepared using 300 mM sodium citrate buffer displaying maximum transfection. The improved transfection potencies of LNP mRNA systems displaying bleb structure can be attributed, at least in part, to enhanced integrity of the encapsulated mRNA. It is concluded that enhanced transfection can be achieved by optimizing formulation parameters to improve mRNA stability and that optimization of ionizable lipids to achieve enhanced potency may well lead to improvements in mRNA integrity through formation of bleb structure rather than enhanced intracellular delivery.

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Development of exogenous loading platforms for functional targeted EV-mediated RNA therapeutics

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One of the greatest challenges for clinical application of RNA therapeutics is effective, nonimmunogenic, and tissue-specific delivery. Due to the intrinsic capability of extracellular vesicles (EVs) to transfer cargo between cells, combined with their low immunogenicity and potential for targeted engineering, they have been leveraged to deliver several types of payloads, including siRNAs.

However, most EV-siRNA loading strategies still have room to improve when it comes to robustness, efficiency, and scalability. Therefore, objective of this study was to develop exogenous EV loading platforms for luminal and surface siRNA loading, aiming for process and loading efficiency, scalability, and EV-mediated potency in vitro and in vivo.

Targeted engineered EVs were generated by transient transfection of CAP cells and purified using flow filtration and FPLC. Modified siRNA conjugated with a Cy5 dye was used for both loading strategies. Electroporation and co-incubation loading conditions were optimised using Design of Experiment (DoE). Loaded samples were further purified to remove unloaded siRNA and characterised using a range of analytical tools.

High loading efficiency was achieved using electroporation (100-200 siRNA molecules per EV) and co-incubation (1000-3000 siRNA molecules per EV). Both methods were scalable, and the process efficiency was improved 60-fold due to DoE parameter optimisation. For co-incubation, siRNA with various conjugations were successfully loaded and showed in vitro potency. Loading by electroporation was enhanced by an active endosomal escape strategy for functional cargo delivery, leading to improved EV-mediated delivery when compared to gymnotic control.

Ultimately, EV-siRNA conjugates were tested for different targeting locations, including brain cells, cancer models and liver, with demonstrated *in vitro* potency and *in vivo* delivery, demonstrating that two exogenous loading platforms were successfully developed for EV-mediated siRNA delivery, paving the way for therapeutic delivery of siRNA using EVs.

P791

Safe SB100X transposase mRNA-mediated co-transfection of human RPE cells with two transposons coding for the neuroprotective factors PEDF and GM-CSF to treat retinal degeneration

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In the therapy approach for treating avascular age-related macular degeneration (aAMD) we are using the *Sleeping Beauty* transposon system combined with electroporation to genetically modify Retinal Pigment Epithelial (RPE) cells to overexpress the neuroprotective, anti-inflammatory, and antioxidant factor combination of Pigment Epithelium-Derived Factor (PEDF) and Granulocyte Macrophage-Colony Stimulating Factor (GM-CSF). Despite an excellent safety profile, it is known that the transposase might have a low cytotoxic effect and a low risk of remobilization of the transposon if supplied as DNA plasmid; the use of SB100X mRNA would be able to prevent both risks and improve the safety profile of the therapy. 50K human RPE cells were transfected directly after isolation ("fresh") or after ≥3 weeks of culture ("pre-cultured") using the Neon® System with 0.03µg of the SB100X transposase DNA/mRNA and 0.47µg of the pFAR4-SB-PEDF and pFAR4-SB-GMCSF, or the pFAR4-SB-Venus plasmid (n=5). Cells were cultured in DMEM/Ham's F12 with 10%-20% FBS until confluence (1% FBS) and analysed for gene expression (RT-qPCR) and protein secretion (ELISA, WB, image-based cytometry [fluorescent protein Venus]) at 3-6 weeks [pre-cultured and fresh] and 3 months [fresh]. The titration of the optimal transposase:transposon ratio, transfection kinetics analyses, and viability determination of the transposase delivered as DNA or mRNA were carried out. The kinetics study performed in the ARPE-19 cell line, showed 100% transfection efficiency (% $Venus^+$ cells) for SB100X DNA and mRNA 24h after transfection; maximal SB100X transposase production was determined at 4±1.20 hs (SB100X-mRNA) and 24±1.7 hs (SB100X-DNA) post-transfection. For double transfected (*PEDF+GMCSF*) pre-cultured RPE cells the fold increase in *PEDF* gene expression (compared to non-transfected controls) was 12.9±11.5 (SB100X-DNA) and 6.4±4.0 (SB100X-mRNA), and for *GM-CSF* 11'877±14'144 (SB100X-DNA) and 6'680±7'823 (SB100X-mRNA); PEDF protein secretion in co-transfected cells compared to controls was 0.84±0.93 (SB100X-DNA) and 0.35±0.31 (SB100X-mRNA) vs. 0.08±0.06 ng/h/10^4 cells, and for GM-CSF 0.10±0.10 (SB100X-DNA) and 0.05±0.05 (SB100X-mRNA) vs. 0 ng/h/10^4 cells. For "fresh" cells, the fold increase in *PEDF* gene expression (compared to non-transfected controls) was 3.3±3.3 (SB100X-DNA) and 1.5±1.1 (SB100X-mRNA), and for *GM-CSF* 1'291±1779 (SB100X-DNA) and 28.9±77.2 (SB100X-mRNA); PEDF protein secretion in co-transfected cells compared to controls was 0.2±0.28 (SB100X-DNA) and 0.2±0.25 (SB100X-mRNA) vs. 0.09±0.04 ng/h/10^4 cells, and for GM-CSF 0.005±0.016 (SB100X-DNA) and 0.0003±0.002 (SB100X-mRNA) vs. 0 ng/h/10^4 cells. The viability study (RealTime-Glo™ Cell Viability Assay, Promega) (n= 4 donors) showed no significant differences between the SB100X-DNA and SB100X-mRNA, neither with the non-transfected controls. Summarized, the SB100X-mRNA is similarly efficient as the SB100X-DNA with similar transfection parameters in co-transfected RPE cells to overexpress PEDF and GM-CSF. The mRNA-transposase is more rapidly produced and thus, transposition is completed earlier. Moreover, the shorter mRNA-transposase half-life (vs. the DNA-transposase) reduces the risk of transposon-remobilization.

P792

Assessing the particle concentration of mRNA-LNP using VIDEODROP : a new parameter to evaluate during LNP process optimization, production and useful for standardization

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LNP (Lipid Nanoparticles) are a leading class of RNA delivery systems, used in the field of vaccines and gene therapy. They are typically composed of a lipid formulation that encapsulates the desired nucleic acid (RNA). A common method for LNP production involves mixing the lipid composition (in the organic phase) with the nucleic acid (in the aqueous phase) using a microfluidic device.

The analytical characterization of these nanoparticles is crucial for ensuring product quality and understanding their *in vitro* and *in vivo* activities.The analytical strategies employed for LNPs include the characterization of the RNA (as the active pharmaceutical ingredient), the lipid composition, and the [mRNA-LNP] nanoparticles. Regarding nanoparticle characterization, the current focus lies primarily on size, polydispersity, and zeta potential. Particle concentration, evaluated through a single particle measurement technique, is an important parameter to monitor for several reasons, including:

- Quality control tests.
- Dosage accuracy.
- Stability assessment.
- Benchmarking studies: When developing new formulations, manufacturing processes, or different storage conditions, quantifying the number of particles allows for effective comparison studies. By comparing particle numbers under different conditions, researchers can assess the impact of the process or composition on transfection performance.

Videodrop is a ready-to-use tool for measuring the particle concentration and size distribution of LNPs in a single drop. Videodrop enables better control of the production process by quickly and easily measuring the concentration. It is the ideal tool to be incorporated into an analytical strategy to assess LNP quality. Moreover, in comparative studies, standardizing cell-based assays using the number of nanoparticles improves the evaluation of LNPs produced under different conditions.

P793

CFTR mRNA co-delivered with the modulator VX809 in lipid-nanoparticles in Cystic Fibrosis cells in air-liquid interface culture induced CFTR protein expression at the same level as normal cells

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Cystic fibrosis (CF) is an autosomal recessive rare disease, caused by mutations in the CF transmembrane conductance regulator (CFTR) gene. Absent or nonfunctional CFTR leads to thick, sticky mucus in the lungs, which results in chronic bacterial infection and inflammation. *In vitro* transcribed mRNA has emerged in the last few years as a new approach for CFTR protein replacement therapy, but problems of delivery must be overcome.

Our aims are to optimise lipid-nanoparticles for human bronchial epithelial cells for mRNA delivery, to deliver CFTR mRNA using lipid-nanoparticles in CF cells to introduce wild type CFTR and to assess the CFTR protein function as chloride channels using Ussing chamber.

We have developed novel receptor-targeted nanocomplex (RTN) formulations consisting of liposomes and receptor-targeting peptide for delivery of CFTR IVT mRNA. We first optimised the RTN formulations, comparing combinations of three cationic liposomes and five peptides for their biophysical properties and transfection efficiency. The optimal formulation achieved almost 100% cellular uptake efficiency and 90% transfection efficiency. Biophysical analysis showed nanoparticles had a size of 130 nm, polydispersity index (PDI) 0.28, charge +38 mV). There were no differences in transfection efficiency between primary normal human bronchial epithelial (NHBE) cells and Cystic Fibrosis Bronchial Epithelial (CFBE) cells. Using the optimised formulation, CFTR mRNA was successfully delivered to NHBE and CFBE cells in submerged culture. In addition, we co-packaged the CFTR corrector VX-809 with CFTR mRNA into RTNs and found that this increased expression of CFTR protein in both NHBE and CFBE cells by approximately 2 to 2.5 times. CFTR protein translated from the IVT mRNA was detected by immunoblotting from 4 hours to at least 48 hours after the CFBE mRNA transfections.

CFTR protein expression was also shown in transfections of ALI culture of CFBE cells by functional analysis of ion transport in an Ussing chamber. After one dose of CFTR mRNA transfection, the change of short circuit current (ΔIsc) in cells transfected with CFTR mRNA was 10-fold larger than in untransfected cells before and after forskolin was added, and it was approximately 83% of normal epithelial cells. Three doses of CFTR mRNA transfections achieved larger ΔIsc than NHBE did after CFTR inhibitor 172 was added into the cells.

CFTR IVT mRNA delivery is a promising novel therapeutic for CF. The flexibility of the RTN formulation allows co-delivery of CFTR mRNA with VX-809, which significantly improved CFTR expression. It can induce the same level of CFTR expression as NHBE in CF cells.

P794

Lipid nanoparticle-mediated messenger RNA delivery for ex vivo engineering of natural killer cells

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Natural killer (NK) cells are part of our innate immune defense and can eliminate both malignant and virally infected cells using their germline-encoded surface receptors. With significantly lower toxicity *in vivo*, NK cells are a popular alternative to CAR-T cells for adoptive cell therapy (ACT). NK cell therapy has shown promising clinical efficacy, yet *ex vivo* engineering to express activating receptors or knock-out inhibitory regulators can further improve their tumor targeting and infiltration. However, delivery of genetic material to NK cells is notoriously challenging. Electroporation, the commonly used non-viral transfection method, has been associated with slow cell recovery and phenotypic changes in NK cells. Alternative delivery strategies are therefore necessary to accomplish efficient NK cell engineering according to regulatory requirements. In this regard, the focus of our research is the mRNA delivery to NK cells via lipid nanoparticles (LNPs) and the assessment of their transfection efficiency and safety profile. To find the optimal LNP formulation for mRNA delivery to NK cells, optimization was performed by altering the cholesterol analog while tuning the ethanol to aqueous volume ratio and the total flow rate parameters of microfluidics. Our findings on the NK cell line KHYG-1 indicate that the replacement of cholesterol with β-sitosterol, not only improves NK cell transfection efficiency from 70% to 84%, but also results in a 38-fold increase in the mean fluorescence intensity (MFI) of eGFP expression. A higher transfection efficiency (93%) and a 4-fold increase in MFI were also achieved by lowering the total flow rate from 11 to 9 mL/min while increasing the ethanol to buffer volume ratio from 1:1.5 to 1:3. In addition, NK cell treatment with the eGFP-mRNA-LNPs maintained cell viability at 99%. Our optimized mRNA-LNP formulation demonstrated superior transfection efficiency on both KHYG-1 NK and Jurkat T cells, when compared to electroporation. Specifically, in KHYG-1 cells, mRNA-LNPs resulted in 92% transfection efficiency compared to 85% of electroporation, with a 35-fold increase in MFI. Likewise, the mRNA-LNPs resulted in 95% eGFP-positive Jurkat cells and a 54-fold increase in MFI, while with electroporation only 63% of the cell population was eGFP-positive. In regard to primary NK cells, cord blood-derived NK cells from Glycostem Therapeutics were successfully transfected with the optimized LNPs with 75% of the cells being eGFP-positive, compared to electroporation, which resulted in 57% transfection efficiency. Taken together, these findings suggest that, through optimization, LNPs are an efficient and non-harmful approach to deliver mRNA for *ex vivo* modification of NK cells, surpassing the transfection capabilities of electroporation.

P795

Molecular chameleon carriers for dynamic RNA delivery

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Continuous chemical evolution has recently resulted in a new class of double pH-responsive nanocarriers for dynamic delivery of various nucleic acid types. These carriers combine the benefits of both polymer- and lipid-based delivery modules and are up to several hundred-fold more potent than previous carrier generations. They contain at least two novel lipo amino fatty acids (LAFs) as cationizable apolar motifs in combination with polar cationizable aminoethylene units of succinoyl tetraethylene pentamine (Stp). The building blocks are connected by lysines via solid phase-assisted peptide synthesis into different topologies (*i.e.*, combs, T-shapes, bundles, and U-shapes) at different ratios of Stp to LAF. The switchable polarity of the LAF is implemented by a central tertiary amine, which disrupts the hydrophobic character once protonated, resulting in pH-dependent structural and physical changes within the carriers, as evidenced by drastic changes in the logarithmic (octanol/water) distribution coefficient logD from around +1 (hydrophobic) at pH 7.4 to -1 (hydrophilic) at pH 5.5. This 'molecular chameleon character' is beneficial for dynamic and fast cargo delivery via both lipopolyplexes and lipid nanoparticles. Screening of different topologies, Stp/LAF ratios, and LAF types identifies tailor-made carriers for the distinct formulations and nucleic acids (including mRNA, and siRNA). Top candidates are characterized by high activity in several cell lines (*i.e.*, various tumour cells, dendritic cells, and macrophages), even in the presence of 90% serum at very low nucleic acid doses. mRNA lipopolyplexes, for example, are still highly potent at ultra-low doses of 3 pg mRNA (~2 nanoparticles/cell), by this being comparable to viral vectors in terms of particle efficiency. Moreover, the carriers display high activity *in vivo* upon systemic application of 1-3 µg luciferase-encoding mRNA in mice, especially in spleen and tumour.

P796

Non-viral delivery of ABCA4 to photoreceptors

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ABCA4-related retinopathies, including Stargardt disease, cone-rod dystrophy, and retinitis pigmentosa, are associated with mutations in the *ABCA4* gene. More than 900 mutations have been described. ABCA4 protein is localized to the outer segments of photoreceptors, and the gene encodes an importer flippase protein that prevents the accumulation of material toxic to the retinal pigment epithelium (RPE). Specifically, mutations in the *ABCA4* gene result in a build-up of lipofuscin, leading to degeneration of the RPE and subsequently, loss of photoreceptor cells. Replacement with wild type ABCA4 protein via gene therapy may slow or block disease progression.

Due to the large size of the coding region (~7 kb), delivery of full length *ABCA4* is not amenable by commonly used adeno-associated virus (AAV) gene therapy platforms. Non-viral gene delivery lacks this cargo size limitation, making it an ideal approach for *ABCA4* gene therapy.

Intergalactic's proprietary non-viral C^{3}DNA allows for the design and delivery of a large DNA payload. Additionally, Intergalactic's proprietary in vivo electro-transfer device, COMET, enables the local delivery of genes to the relevant cell type. Our goal was to assess the feasibility of COMET-mediated delivery of non-viral C^{3}DNA expressing the full length human *ABCA4* gene to adult porcine and non-human primate (NHP) retinas.

We generated covalently closed and circular DNA (C^{3}DNA) for codon optimized full-length human *ABCA4* and delivered it subretinally to adult porcine or NHP eyes followed by COMET application. We assessed *ABCA4* mRNA expression using reverse transcription followed by

quantitative PCR (RT-qPCR) or using droplet digital PCR (ddPCR). Transgene-derived ABCA4 protein was detected by immunohistochemistry (IHC) using an antibody specific to human ABCA4 protein.

In the porcine eye, human *ABCA4* mRNA and protein expression were detected by RT-qPCR and IHC, respectively, at 5-6 days post-treatment. Dual labeling studies further identified the cellular and subcellular location of transgene-derived ABCA4 protein to be primarily in the photoreceptor outer segments. Importantly, the expression pattern of C^{3}DNA-derived human ABCA4 protein mimicked that of the endogenous protein.

Subretinal injection requires ocular surgery that is not amenable to frequent dosing, therefore it was critical that we demonstrate durability of expression. We conducted a series of persistence studies in porcine retina to assess the durability of human ABCA4 protein expression. After one single subretinal injection of C^{3}DNA-*ABCA4*, human ABCA4 protein was readily detected in the outer segments of photoreceptors at 1, 3, 6 and 12 months, the longest time point evaluated to date. In the NHP retina, *ABCA4* mRNA levels were detected in the retina 6 months post-single administration of C^{3}DNA-*ABCA4* and COMET. Collectively, these data support long term persistence of C^{3}DNA-derived *ABCA4* in porcine and NHP retinas using Intergalactic's non-viral C^{3}DNA plus COMET gene delivery platform.

We have demonstrated feasibility of COMET-mediated delivery of non-viral C^{3}DNA expressing the full length human *ABCA4* gene to relevant cell types in the retina and demonstrated the expression of human ABCA4 protein that persists at least to 12 months post-treatment in adult porcine retina. Our data also demonstrate 6 months durability of transgene-derived *ABCA4* mRNA expression in NHP retina.

P797

Lipid nanoparticles as vector for delivery of CRISPR-Cas9 targeting *RUNX1-RUNX1T1* in AML with t(8;21)(q22;q22.1)

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Acute myeloid leukemia (AML) with t(8;21)(q22;q22.1) resulting in the fusion oncogene *RUNX1-RUNX1T1* is a well-described subtype of AML. Generally perceived as associated with a favorable prognosis, the main cause of mortality in these patients remain relapse, occurring in an estimated 30% of patients. Gene editing technology CRISPR-Cas9 allows for induction of specific dsDNA breaks inducing premature stop codons or major DNA damage, resulting in disruption of its target. Previous research has established that an intron-targeting dual-gRNA approach can disrupt the *RUNX1-RUNX1T1* fusion oncogene, leading to inhibited leukemic cell growth and proliferation, suggesting its potential as a future therapeutic in the treatment of AML. In this study, we investigated lipid nanoparticles (LNPs) as a potential vector for delivery of gene therapy for AML. Human AML cell line Kasumi-1, *RUNX1-RUNX1T1* positive, was used for *in vitro* experiments with THP-1 cells, *RUNX1-RUNX1T1* negative, used as control. Primary patient bone marrow mononuclear cells (BM-MNCs) from 1 patient with AML and 1 patient with an undiagnosed myeloid malignancy, both treated at our department were used.

LNPs were synthesized from SM-102 or D-lin-MC3-DMA, DSPC, Cholesterol and DMG-PEG2000 in molar ratio 50:10:39:1, and assembled using Nanoassemblr™ Ignite®. The LNPs were packed to contain: (i) GFP-mRNA; (ii) gRNA; or (iii) Cas9-mRNA. Flow cytometry was utilized for analysis of GFP expression and evaluation of transfection efficiency of LNP-GFP 48 hours following treatment of cells. PCR was used for evaluation of cleavage at the target site in Kasumi-1 and THP-1 cells treated with our dual-gRNA and Cas9-mRNA approach. High GFP expression was detected in both THP-1 and Kasumi-1 cells corresponding > 90% expression. Treatment of primary patient BM-MNCs demonstrated LNP capacity for transfection of both monocytes, granulocytes as well as leukemic blasts, with transfection of blasts and granulocytes only occurring using LNPs composed with ionizable lipid D-lin-MC3-DMA. Curiously, no GFP expression was detected in the lymphoid compartment following LNP transfection. Treatment of Kasumi-1 cells with CRISPR technology targeting the *RUNX1-RUNX1T1* fusion gene resulted in consistent disruption of the fusion gene at various dosages when delivered in LNPs. Collectively, our data indicates that LNPs are potential vectors for delivery of CRISPR-Cas9 technology to myeloid cells including malignant leukemic blasts. Furthermore, we have demonstrated that LNPs can deliver our dual-gRNA and Cas9-mRNA approach to Kasumi-1 cells and successfully disrupt the fusion gene target. Collectively, our results suggest a future role of LNPs for *in vivo* delivery of gene therapy for treatment of AML.

P798

Membrane recruitment and calcium-dependent endosomal release of a phospholipase for enhanced DNA transfection

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Non-viral gene therapy has the potential to battle diverse diseases. However, the development towards efficient non-viral gene therapy still faces many hurdles, including poor endosomal escape. Our aim is to increase the recently reported non-viral transfection system termed TFAMoplex. The latter is based on the human mitochondrial transcription factor A (TFAM) and includes a phospholipase C (PLC) for enabling endosomal escape. In this work, we aimed at developing a system to recruit PLC to the plasma/endosomal membrane and to release it from the TFAMoplexes once inside the endosomal compartment in order to increase the endosomal escape and therewith transfection efficiency. Membrane recruitment of PLC was achieved by genetically fusing PLC to a nanobody targeting the ubiquitously expressed tetraspanin CD9 (anti-CD9nb) with high binding affinity. Controlled release of the PLC inside the endosomal compartment was achieved by using a calcium-sensitive split protein. Calcium-dependent association and dissociation of the two split parts was assessed by split nano luciferase (nanoLuc) complementation. To release the PLC in the endosomal compartment under low calcium concentrations, one part of the calcium-sensitive split protein was genetically fused to PLC and anti-CD9nb. The second part was genetically fused to TFAM, allowing the non-covalent coupling of the two proteins *via* the calcium-sensitive split proteins. Transfection efficiency was assessed by transfecting HeLa cells with a reporter plasmid and quantified by flow cytometry analysis. The split nanoLuc complementation assay indicated that the two calcium-sensitive split proteins formed a heterodimer at calcium concentrations in the high micromolar and low millimolar range. Association also occurred in serum. No significant association could be detected under low micromolar calcium concentrations neither at neutral nor acidic pH. TFAMoplexes containing anti-CD9nb for membrane recruitment and the calcium-sensitive PLC release system resulted in a

30 fold increase in transfected cells compared to the control system lacking the anti-CD9nb and calcium-sensitive linkage. Membrane recruitment mediated by anti-CD9nb together with employing a calcium-sensitive split protein to release PLC improved transfection efficiency. This novel system is simple as well as effective and could be readily translated to other gene delivery platforms. 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).

P799

Efficient and gentle non-viral engineering of therapeutic cells by photoporation

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Engineering of cells for cell-based therapies requires intracellular delivery of effector molecules like siRNA, mRNA, pDNA or gene-editing nucleases. Although substantial effort has gone into developing viral and non-viral nanocarriers, they each come with their limitations, including safety concerns and limited efficiency. Physical delivery methods offer an interesting alternative solution. Electroporation is a notable example but is often associated with high cell toxicity. Photoporation, which combines laser technology with photothermal nanosensitizers, has emerged as a promising intracellular delivery method combining high delivery efficiency with flexibility and excellent cell viability. In our research we have demonstrated that photoporation can be successfully used to deliver a broad variety of effector molecules (mRNA, siRNA, Cas9 RNPs, nanobodies, contrast agents …) into many different cell types, including hard-to-transfect cells such as lymphocytes and stem cells. [1] We have also demonstrated the unique capability of photoporation to perform spatially controlled cell transfections, even with single-cell precision. For the production of engineered therapeutic cells, photoporation can be performed with biodegradable polymeric photothermal nanoparticles [2] or biocompatible photothermal nanofibers to obtain a nanoparticle-free cell product [3]. Compared to electroporation we have demonstrated that photoporation is much gentler to cells, avoiding unintended phenotypic or functional changes. For instance, using nanofiber photoporation to downregulate PD-1 expression in human CAR T cells, their cytolytic potential was improved as compared to electroporated cells, resulting in faster tumor regression in vivo. In conclusion, photoporation has proven to be a promising next-generation non-viral cell transfection technology for the production of engineered therapeutic cells.

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P800

bZIP Protein Dimerization in a TFAM-Based Transfection System for Efficient and Stable Gene Therapy

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Gene therapy holds great promises in medicine and biotechnology and is already providing new treatments for diseases. Due to its large size and negative charge, DNA requires carriers to be efficiently delivered into the target cells. Our group recently developed a gene delivery system based on the human protein mitochondrial transcription factor A (TFAM), a key organizer of mtDNA. *In vitro*, TFAM binds and condenses DNA into nanoparticles, which we named TFAMoplexes. After fusion with functional proteins like phospholipases or kinases, the resulting DNA-protein complexes can escape from the endosomal compartment, thereby enabling efficient transfection. In this study, we further enhance the transfection potential of TFAMoplexes by incorporating basic-leucine-zipper (bZIP) domains. We make use of their DNA binding capability that occurs after dimerization for increased complex stability and transfection efficacy.

We challenged our system by selecting an incubation time of only 10 min, halving the quantity of DNA used, and transfecting cells in full fetal bovine serum. Transfection levels doubled compared to the original TFAMoplex without bZIPs, with over 40% of cells successfully transfected. The short incubation time and low quantity of DNA did not significantly affect transfection efficacy *vs*. more favorable conditions, indicating robust performance under a range of conditions. While the original TFAMoplex already outperformed the commercial transfection agent XtremeGene™ by ten-fold, the addition of the best performing bZIP protein BACH1, achieved a twenty-fold increase compared to this control. In conclusion, this study presents a promising approach for improving gene delivery, with potential wide-ranging applications in gene therapy.

P801

Safety of a Sleeping-Beauty-mediated gene therapy to treat age-related macular degeneration: exclusion of toxicity in rabbits transplanted with allogenic PEDF-transfected IPE cells

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With more than ten therapies that got marketing approval, gene therapy is becoming part of clinical routine. However, the majority of approved therapies are treating monogenic rare diseases or cancer. The treatment of complex diseases which are influenced by multiple genetic and non-genetic factors remains challenging. Age-related macular degeneration (AMD) is such a complex disease influenced by age, environmental factors and genetic predispositions. It is the leading cause of blindness in elderly patients in industrialized countries and has been referred as time-bomb in coming years due to aging populations and related increased numbers of patients.

Current gold-standard to treat the neovascular form of AMD (nAMD) are approx. monthly repeated intra-ocular injections of anti-VEGF molecules. Integrative gene therapy approaches might reduce costs of this chronic therapy, improve patients' compliance, and reduce risks of side effects. We developed an approach to halt macular neovascularisation in nAMD by subretinally transplanted iris pigment epithelial (IPE) cells transfected using the Sleeping Beauty transposon system and electroporation with the pigment epithelium-derived factor (PEDF) gene.

Transplants were prepared from allogenic IPE cells isolated from normal rabbits, cultured until confluence in complete medium (DMEM/Ham's 12, 10% FBS, 1% Penicillin/Streptomycin, 1% Amphotericin B). Then, 50,000 IPE cells were mixed with the pFAR4-T2-SB100X transposase (0.03µg) and the pFAR4-T2-PEDF or the pFAR-T2-Venus transposon (0.47µg) plasmids and electroporated using the Neon® electroporation system. Toxicity was evaluated in ≥3 months old normal Chinchilla Bastard rabbits (both sexes) transplanted with 20,000 IPE cells suspended in 30 µL BSS-Plus. The cells were transplanted subretinally into the right eye (n=3-5 rabbits/group); the left eye served as control (non-treated; sham-treated with Venus-transfected cells, a yellow-fluorescing marker protein). Animals were followed for 1h-3 months regularly evaluating welfare, blood, intra-ocular pressure (IOP), fundus and retina (ocular coherence tomography, OCT). After sacrifice, retinas were analysed (immuno-)histologically (hemalum/eosin, Iba-1, GFAP, Caspase-3, CD68, rhodopsin, RPE65, CaB-5, MAP-1/2, Ki-67, Pancytokeratin, Venus, PEDF, TUNEL).

All 32 treated rabbits showed excellent welfare (score: 9.84±0.37 from 10; weight: Δ0.87±0.41 kg [0d-3 months]) and normal IOP; right: 11.32±2.17 (0d) and 12.11±2.26 mmHg (3 months); left: 11.65±2.39 (0d) and 12.00±2.40 mmHg (3 months). The mean of all 37 measured blood values (hepatic, renal, inflammation, necrosis, mineral parameters, differential blood count, and erythrocyte indices) was in the reference range at all time points (e.g., AST: 12.54±13.27 U/l, urea: 6.97±0.25 mmol/l, CK: 217.8±30.15 U/l, Calcium: 3.59±0.03 mmol/l, lymphocytes: 2.89±1.29 G/l, MCHC: 31.10±1,23 g/dl [7d]). Fundoscopy and OCT visualized successful transplantation by the retinal bleb that reattached within 7d. Low post-surgical complication rates were determined (0.5% of haemorrhage, 0.5% of fibrous scars at the injection site, 7.4% of retinal atrophy in the region of artificial retinal detachment); no side effect due to the cell product was detected. Tissue analysis retrieved the transplant (Venus-transfected cells), excluded inflammation, apoptosis, tumorigenicity, and confirmed normal morphology.

Data confirmed excellent local and systemic tolerability of PEDF-transfected IPE cells transplanted subretinally corroborating the hypothesis that the transplantation of autologous PEDF-transfected IPE cells can be safely performed in the planned phase Ib/IIa clinical trial to treat nAMD patients.

P802

Understanding the endosomal escape of EV-delivered therapeutic molecules

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Extracellular vesicles (EVs) have a high therapeutic potential as efficient carriers of biomolecules with low immunogenicity. After endocytic uptake, most EV cargoes need to be released into the cytosol through endosomal escape to exert their function. One proposed mechanism to improve

endosomal escape involves the expression of fusogen peptides on EV membranes, which fuse with the endosomal membrane and allow EV contents to be released into the cytosol. In this context, EV engineering with the Vesicular stomatitis virus glycoprotein (VSV-G) has been shown to significantly increase cargo delivery capability. However, the introduction of a viral peptide could trigger a pro-inflammatory immune response. Ascertaining the minimal amount of VSV-G required for efficient endosomal escape would therefore enable improved engineering of EVs that retain a good delivery profile whilst minimising toxicity.

In this study, we produced EVs containing different quantities of VSV-G and analysed the minimal dose of VSV-G that improves endosomal escape *in vitro*. Moreover, we generated a VSV-G-inducible stable cell line to regulate VSV-G expression levels in EV producing cells. Proof-of-concept studies showed that VSV-G-mediated siRNA delivery can be achieved in vivo and in vitro, leading to successful knockdown of target genes. This work represents a step towards the use of fusogen peptides to enhance cargo delivery while minimising the immune response.

P803

circVec: a versatile circular RNA vector cassette for enhanced and durable intra-cellular protein expression

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Circular RNA (circRNA) is a recently discovered class of RNA expressed endogenously by eukaryotes. In contrast to mRNA, circRNA is resistant to exo-nucleolytic decay, which results in high intra-cellular persistence. The current understanding of circRNA biogenesis and functionality has improved significantly, including recent engineering of circRNAs for highly efficient translation of proteins. Due to their beneficial intrinsic characteristics, circRNA is emerging as a potential therapeutic class that may overcome several challenges facing mRNA-based therapies.

circVec is a novel spliceosome-dependent circRNA expression system, designed for durable, high yield vector-based circRNA biogenesis. While circRNAs are not *per se* templates for endogenous translation, they can be engineered to drive highly efficient protein expression through the insertion of internal ribosomal entry sites (IRES). Using a panel of proof-of-concept-reporters, we show that certain design-features within the circRNA sequence itself, as well as in the flanking regions, strongly influence both circRNA biogenesis and protein expression. To further optimize expression, 1000+ IRES elements were screened across multiple cell lines to identify the shortest and most potent IRES sequences to drive circRNA-based protein translation.

Furthermore, we show that optimized circVec designs outperform conventional mRNA-based expression cassettes for both protein yield and durability of expression. The circVec system is versatile and transferable to a variety of vector types, and may enable optimization of several conventional and emerging gene delivery and protein expression platforms. As such, circVec represents a promising new platform for vector-based protein expression, with broad applicability in various therapeutic areas requiring potent and durable transgene expression, including monogenetic disorders, infectious disease and oncology.

P804

Engineered virus-like particles (eVLPs) for efficient in vitro delivery of precise gene editing therapy

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Recent developments in the precise gene editing field enabled the correction of many different genetic diseases in vitro and in vivo. For therapeutic application, safe and efficient delivery methods of these tools are required. Virus-like particles (VLPs) are DNA-free vesicles taking advantage of viral delivery proteins while bypassing the risk of genomic integration and long-term expression. Here, we produce and optimize engineered virus-like particles (eVLPs) as a tool for in vitro delivery of base and prime editing machinery in the form of ribonucleoproteins (RNPs). We showed highly efficient base editing in HEK293T cells and in intrahepatic cholangiocyte organoids (ICOs) after delivery with eVLPs. Furthermore, we adapted the system for the delivery of prime editors and showed editing in HEK293T cells. Further optimizations of the PE-eVLP system allowed us to increase the editing efficiencies with prime editing by 16%. Finally, cystic fibrosis colon organoids were chosen to show functional correction after delivery of editing tools, which did not result in editing of the targeted mutations. Altogether, these results support eVLPs as an efficient in vitro delivery system for precise gene editing machinery.

P805

Application of CRISPR/Cas12a technology in the edition and detection of pathogenic mutations

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CRISPR/Cas technology has meant a breakthrough in the gene-editing field. This system comprises both DNA and RNA-targeting proteins. Cas9 and Cas12a are the most widely used DNA-targeting nucleases. To date, most research has focused on Cas9, however, Cas12a possess intrinsic features that make them highly attractive for *in vivo* applications. Moreover, Cas12a displays an additional *trans* nuclease activity that can be exploited for the detection of DNA/RNA sequences. This project aims to explore the potential of the CRISPR/Cas12a system for the diagnosis and the treatment of genetic diseases.

The difficulty to deliver CRISPR components constitutes a major challenge limiting their therapeutic potential. So far, viral methods are the preferred approach for CRISPR delivery. However, they entail important drawbacks such as low loading capacity and immunogenicity. Nanotechnology constitutes an interesting alternative to overcome these obstacles. In this work we have used iron oxide magnetic nanoparticles for the intracellular delivery of Cas12a. MNP-Cas nanocomplexes were efficiently internalised by different cell lines and, once internalised, resulted in gene editing rates comparable to those obtained with commercially available delivery agents.

Additionally, we have assessed Cas12a's potential to detect oncogenic single-base mutations. Specifically, we have focused on *GNAQ*, a gene that is frequently mutated in uveal melanoma. We have been able to use Cas12a to discriminate between wild type and mutated sequences

of *GNAQ* in plasma samples from uveal melanoma patients which paves the way for the design of sensing tools for simpler and cheaper non-invasive diagnosis.

P806

Electro-Transfer Enabled Non-Viral, In Vivo Gene Delivery

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We have developed a novel platform enabling efficient *in vivo* gene delivery across a variety of target tissues in multiple species. The platform, named COMET (Cellular delivery Of genetic Material by Electro-Transfer), is based on the principle of electro-transfer in which a low energy electric field transiently permeabilizes the cellular membrane, providing a pathway for gene transport into the cell and nucleus. COMET consists of three key elements: low energy generators, specific waveforms and algorithms, and electrodes designed to access specific tissues. Preclinical experiments have demonstrated successful cellular transfection with large gene constructs (up to 12 kb tested), far exceeding the 4.7 kb limit of AAV-based gene delivery platforms, and enabling gene replacement approaches even when mutations affect large genes, such as *ABCA4*-associated.

COMET-mediated delivery of covalently closed and circular (C^3) DNA encoding green fluorescent protein (GFP) has been tested and demonstrated in multiple species and in multiple tissues: mice (brain, eyes, liver, kidney, tumors), rats (skin, joint cartilage), pigs (eyes), and non-human primates (NHP, eyes).

Intergalactic utilized the COMET platform to develop a custom generator, algorithms, and electrodes for treating *ABCA4*-related retinopathies that is characterized by progressive loss of central vision. Leveraging well established subretinal delivery techniques, initial experiments were conducted to deliver C^3GFP with COMET to adult porcine and NHPs. A solution of C^3GFP was injected in the subretinal space, via a flexible-tipped cannula driven by the viscous fluid control unit, standard on vitrectomy consoles. A COMET electrode was then inserted into the retinotomy and COMET energy was applied, enabling transfection. Results demonstrate 12-months of persistence of GFP expression (longest time point tested).

In vitro testing was also conducted to assess the subcellular localization of electro-transfer-mediated delivery of C^3DNA using human cell lines. These results indicate that electro-transfer results in a preferential translocation of C^3DNA into the nuclear compartment and consequently results in reduced immune activation as compared to other non-viral delivery methods.

Collectively, our results suggest that COMET is a safe and efficient non-viral DNA delivery platform, with the potential to address significant limitations of other delivery systems, both viral and non-viral.

P807

Bridging treatment modalities with stable, non-viral gene transfer of synthetic AAV genomes delivered by lipid nanoparticles

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Virus-based gene therapies with AAV vectors brought stunning success in an increasing number of clinical settings, but concerns regarding safety and patient access prevail with no clear path to resolution. Thus, the focus is broadening toward non-viral gene transfer fostered by the triumph of mRNA/lipid nanoparticle (LNP) vaccines that have safely delivered nucleic acid material to billions of people. However, LNPs are currently not used for gene replacement therapy, the long-lasting treatment modality for genetic disorders, and the strength of AAV-based therapies. The applicability of AAV vectors for gene replacement therapy is enabled by the episomal stability of AAV genomes mediated by their inverted terminal repeats (ITRs). On the other hand, safety concerns are primarily associated with the immunogenicity of the capsid at high doses, a potentially heterogenous genetic payload, and limitations to patient access due to low productivity in cell culture. Here, we describe the production of synthetic AAV genomes, termed Aavecule® DNA, and their packaging in lipid nanoparticles as a possible bridge between treatment modalities that preserves the unique advantages of the AAV genome while allowing a highly scalable vector production from chemically defined base materials. Aavecule® synthetic AAV genomes encoding different fluorescence reporters were produced by a modified Rolling Circle Amplification process and structurally resembled one polarity of the linear ssDNA genomes of AAV vectors. Genomes were then packaged in LNPs based on the ionisable lipid SM-102. Encapsulation reached an efficiency >90% in a nuclease protection assay, and the resulting particles were highly homogenous at around 170 nm with a polydispersity index <0.1. Next, we optimised the Aavecule®-LNP manufacturing procedure using a DoE approach, modifying the lipid composition and other process parameters. The resulting particles transduced model cells with a transfection efficiency of >90% while not impairing the viability of the culture. Since the utilised lipids are optimised for cytosolic delivery, we hypothesise that the ITRs may possess intrinsic nuclear localisation signals making the fully synthetic approach viable. The approach also releases constraints on ITRs imposed by AAV biology in cell culture. Future work can therefore explore the ITR design space more freely with regard to the strength and stability of gene expression, ITR immunogenicity, and other factors currently discussed in the AAV gene therapy field, like unintended vector genome integration.

P808

Assessment of toxicity in retinal organotypic culture using PEDF/GM-CSF as therapeutic proteins in a gene therapy approach to treat AMD

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Various gene therapy strategies are currently being explored as potential therapeutic options for ocular conditions such as age-related macular degeneration (AMD). We suggest a non-viral *Sleeping Beauty* (*SB100X*) transposon system-based gene therapy to treat AMD by transplanting Pigment Epithelium-Derived Factor (PEDF)- and Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)- transfected iris pigment epithelial cells in the eye. Testing these cell products in relevant cell types is crucial for obtaining accurate and meaningful data regarding their efficacy, safety, mechanism of action, and potential clinical application. This study aimed to evaluate the toxicity of the PEDF and GM-CSF proteins, individually and in combination, in a porcine retinal organotypic culture system. Adult porcine neurosensory retina isolated from eyes obtained from local slaughterhouse 5-8 h post-mortem was cultured in serum free Ames' medium for 14 days supplemented with growth factors (1% N2 and 1% B27); recombinant proteins (PEDF and GM-CSF, 500 ng/mL) were added freshly to the medium every day. Retina samples were analysed at different time points, i.e., day 1, 6, 9, and 14. Viability of retinal cells was evaluated by the CytoTox-Glo® assay. Hemalum and eosin staining was performed to examine the structural integrity of retinal layers and to measure retinal thickness. Immunofluorescent staining was used to determine potential inflammatory effects (GFAP=Glial fibrillary acidic protein) and cell specific degradation (Rhodopsin) in retinal tissue. Treatment with PEDF, GM-CSF or the combination did not lead to any structural abnormalities in the retinal layers with comparable retinal thickness($p \geq 0.05$) in all the groups at all time points (Day 1: Control=51.409±4.40 µm, Day 6: Control 58.68±7.90 µm, PEDF=49.97±11.59 µm, GM-CSF=47.97±5.99 µm, PEDF + GM-CSF=65.08±1.76 µm, Day 9: Control=68.12±14.87 µm, PEDF=70.43±11.74 µm, GM-CSF=56.68±12.66 µm, PEDF + GM-CSF=65.96±18.18 µm, Day 14: Control=51.01±10.47 µm, PEDF=64.12±9.52 µm, GM-CSF=47.65±8.04 µm, PEDF + GM-CSF=43.73±8.14 µm,). No significant difference was observed in comparing the viability of treatment groups with the control group measured by the release of a "dead cell protease" into the medium (PEDF=2420±2718 AU, GM-CSF=1457±2174 AU, PEDF + GM-CSF= 1683±2167 AU, Control=1734±2718 AU, P = 0.8566); the lower the luminescence measured in AU, the higher the viability. A trend for a better photoreceptor outer segment preservation in PEDF and GM-CSF-treated retinae was observed by immunohistological anti-rhodopsin staining. GFAP staining of muller cells showed only low inflammation. In summary, PEDF and GM-CSF proteins given at a concentration of 500 ng/mL, individually and in combination, do not induce any toxic effect in porcine retinal organotypic culture and are well tolerated in retinal tissue. The combination of PEDF and GM-CSF has shown a trend for an improved photoreceptor outer segment preservation possibly because of a synergistic effect of both proteins involved, supporting their efficiency as therapeutic agents to treat AMD.

P809

Amphiphilic Lipopeptide-based Nanocomplexes as a Non-viral Delivery System for CRISPR-Cas9 Ribonucleoprotein

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Clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) exhibits promising potential in the realm of genetic diseases as it enables gene disruption through nonhomologous end joining (NHEJ) or gene correction through homology-directed repair (HDR).

However, the effective transport of CRISPR components and concurrent delivery of homology-directed repair DNA into the target cell nucleus pose ongoing challenges. As a result, the clinical application of CRISPR technology faces hindrances due to the limited availability of delivery systems that can ensure both safety and efficiency. In the present study, we formulated and characterized a series of cell-penetrating peptides (CPPs) that have been chemically modified with fatty acids, namely Octanoic acid, Linoleic acid, Oleic acid, and Stearic acid. The main objective of this modification was to facilitate the efficient intracellular delivery of Cas9 ribonucleoprotein (RNP) and HDR template molecules, The fatty acid-conjugated peptides exhibited notable stability when complexed with both RNP and RNP/HDR template, as evidenced by long-term dynamic light scattering (DLS) measurements. Notably, among the various fatty acid modifications, the Oleic acid-conjugated peptide displayed the highest stability when forming nanocomplexes with RNP and a RNP/HDR template. Additionally, all the fatty acid-modified nanocomplexes, encompassing RNP-CPP and RNP/HDR-CPP, demonstrated robust capabilities for gene editing and gene correction, surpassing the performance of commercially available reagents such as CRISPRMAX. The specific fatty acid composition within the peptides was found to exert a pivotal influence on the efficiency of gene editing and gene correction. Notably, among the various fatty acid-modified peptides, the presence of Oleic acid-modified CPP and RNP resulted in the formation of the most stable nanocomplexes and demonstrated the highest levels of gene editing and gene correction efficiencies across multiple reporter cell lines. Moreover, the nanocomplex comprised of Oleic acid-modified-CPP-RNP exhibited notable resilience against trypsin, effectively safeguarding the encapsulated Cas9 protein cargo. The utilization of CPPs as a delivery strategy not only offers considerable prospects for achieving efficient delivery of Cas9 and RNP across diverse cell types, but also exhibits substantial potential for enabling the delivery of other gene-editing proteins in forthcoming applications. Moreover, this technology has the potential to amplify and expedite the progress of CRISPR-based gene therapy applications.

P810

Improved methods for CRISPR editing and Homology-Directed Repair (HDR) in iPSC

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CRISPR-based editing and homology-directed repair (HDR) has become a widely used tool for introducing precise changes in the genome. However, it's utility in various cell types such as iPSC is often impeded by the lack of reliable reagents and protocols. Here, we report optimized CRISPR reagents and design parameters to improve HDR in various cell types including iPSC. Using Alt-R™ S.p. Cas9, Alt-R guide RNAs and Alt-R donors oligos, we present efficient and precise insertion of donor template at multiple target sites measured by Next Generation Sequencing (NGS). HDR rates can be further boosted by the use of small molecule compound, Alt-R HDR enhancer V2. In addition, we present the performance of an improved small, protein-based HDR enhancer as an additional reagent to increase HDR rates when co-delivered with Cas9 RNP complex. We also feature rhAmpSeq™ CRISPR Analysis System that provides a streamlined workflow for high throughput, targeted NGS based screening of editing outcomes and HDR rates in any cell type of interest.

P811

Quantifying and improving the cellular uptake of the TFAMoplex gene delivery system

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Non-viral delivery of DNA into human cells often lacks an efficient method of cellular uptake. The latter can be increased via the addition of protein-based uptake factors to the transfection system. We previously developed a promising non-viral vector based on human mitochondrial transcription factor A (TFAM). TFAM is able to compact plasmid DNA into approximately 100 nm complexes (TFAMoplex). Various effector proteins were fused to TFAM to achieve endosomal escape and cytoplasmic mobility. However, the cellular uptake of the system remained low. In this work we aim to enhance the endocytosis of TFAMoplexes by conjugating uptake enhancing proteins to TFAM.

Receptor targeting moieties of human origin were attached to the DNA carrier. Different proteins, such as antibody fragments, growth factors and glycolipid binding motifs, were investigated. A highly specific split calbindin system that couples two protein fragments in the presence of Ca^{2+} with high affinity was used for modular conjugation to TFAM. The uptake factors were investigated in two assays. First, by direct uptake quantification and second, by measuring their effect on transfection efficacy. Flow cytometry was used to quantify the internalization of a panel of 10 receptor ligands, tagged with an mCherry fluorophore. The effects of Pitstop 2 and β-cyclodextrin on transfections, were assessed following established protocols for endocytosis inhibition. Transfection efficacy was measured by flow cytometry of eGFP reporter expression after 24h.

Flow cytometry experiments of labeled uptake factors revealed a positive correlation between ligand concentrations ranging from 10 pM to 100 nM and internalized signal. It could be shown that lipid-raft mediated endocytosis serves as the most productive uptake pathway for transfections with this system, since its inhibition led to almost complete abrogation of transfections. Inhibition of other routes had no effect. Increasing ligand concentration beyond a certain threshold led to diminished transfection efficacies, from 30% to less than 2% GFP positive cells, indicating a possible lack of access of the ligand to its receptor.

In conclusion, insights into the use of receptor targeting moieties for improved DNA uptake were obtained. Additional work is needed to understand how targeting ligands should be positioned of the TFAMoplex surface to promote uptake.

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P812

QbD-based evaluation of disulfide bond poly (amido amine) (PAA) nanoparticles as suitable nanocarriers for mRNA delivery

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Disulfide bond poly (amido amine) (PAA) nanoparticles (NP) are composed of cationic and bio-reducible polymers and are promising nanocarriers for mRNA delivery. This study was an attempt to further improve the transfection efficiency of these PAA NPs, at lower mRNA concentrations and with low cytotoxicity. The PAA NPs were tested in 3 different cell lines, namely Human embryonic kidney cells (HEK293-commonly used and easy to transfect), human chondrocytes (C28/I2) and hard to transfect cells T-lymphocytes (Jurkat). The experimental conditions for achieving maximum cell viability (CV) and transfection efficiency (CT) of the PAA NPs with β-gal mRNA were determined by following the Quality by Design (QbD) approach.

QbD approach was initiated by performing a F*ailure Modes Effects Analysis* (FMEA) to identify the critical factors. It indicated the type of polymer, the polymer-to-genetic payload (P: G) and the dosage of mRNA (D) used were the critical factors for which a *screening design* of experiments was performed. In this study, 3 different PAA polymers with different functional groups (PAA1, PAA2 and PAA3) were compared aiming at determining the best polymer for formulating the NPs. The best PAA polymer, the range of ratios of the genetic material to the NP and dosage of the NPs were then selected for the optimisation experiments. Then, a *D-optimal optimisation design* was performed independently for three different cell lines HEK-293, C28/I2 and Jurkat cells with the selected PAA NPs.

The outcome of the screening study suggested that the P: G ratio has less significance in the transfection of the cells when compared to the type of the polymer chosen, and the dosage of the mRNA which have shown significant variations in the transfection rates. The cell transfection was inversely proportional to the P: G ratios and directly proportional to the dose of the mRNA. The coefficients plot indicated that NPs made with PAA2 showed higher levels of cell transfection when compared to NPs of PAA1 and PAA3. So, NPs of PAA2 were tested on all three cell lines using the D-optimal design. The optimisation design inferred that the P: G ratio at 10:1 to 18:1 and the dosage of administered RNA from 408 to 480 ng, gave the best transfection rates. These design space results were then validated by performing the transfection experiments in the conditions indicated by the optimal set point (P: G ratio: 10:1 and RNA dosage of 432 ng). The cell viability and transfection results were found to be accurately predicted by the model to give the highest average cell transfection and viability of HEK293 cells (CV- 103.13% and CT – 3670.8 µ units/ug), Jurkat cells (CV- 76.9% and CT – 302.44 µ units/ug) and C28/I2 cells (CV- 100.54% and CT – 2126 µ units/ug).

The design space of the P: G ratio and the dosage of the NPs required for higher cell transfection rates in the three different cell lines was successfully determined. The PAA NPs that were formulated from the optimum runs could also transfect the hard-to-transfect cells like the Jurkat cells with less toxicity.

P813

Endogenous DNA-binding proteins for intracellular visualization of exogenous DNA

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Efficient cytoplasmic DNA mobility is a key bottleneck in non-viral gene delivery. Yet, little is known about the intracellular fate of the delivered genes since the transfected DNA is difficult to visualize in sufficient precision inside the cytoplasm. Current intracellular visualization systems for transfected DNA generally suffer from artefacts, photobleaching, and high background signal.

We make use of two endogenous DNA-binding proteins to specifically track DNA in the cytoplasm. One is the cAMP Response Element-Binding Protein (CREB). The DNA binding site of CREB is present in five repeats on the viral CMV promoter incorporated in common transfection plasmids. CREB might play an important role in the translocation of cytoplasmic DNA by binding the CMV promoter and pulling the DNA into the nucleus via its nuclear localization signal. The other DNA-binding protein we utilize is the barrier-to-autointegration factor (BAF) that clusters exogenous DNA in the cytoplasm. To date, BAF is mainly visualized by overexpressing GFP-BAF. We designed a BAF cluster staining method by introducing split-luciferase parts at the N-termini of each BAF monomer. Only in BAF clusters, the split-luciferase parts are sufficiently close to emit light upon substrate addition.

We engineered HeLa cell lines stably expressing EGFP-labeled CREB or split-luciferase BAF, first, via stable insertion at genomic pseudo AttB sites and second, by genetically fusing the EGFP or split-luciferase gene to the respective genomic locus using CRISPR/Cas9. With these cell lines we tracked the delivered DNA bound to EGFP-CREB or split-luciferase BAF by confocal laser scanning microscopy.

EGFP-CREB was found to localize in the nucleus. Colocalization with labeled DNA and Hoechst staining showed that EGFP-CREB also binds to the transfected DNA in the cytoplasm. HeLa cells expressing split-luciferase BAF showed minimal background of BAF dimers. Light emitting clusters close to the nucleus were detected after transfection.

With these new tools for intracellular DNA visualization, the efficiency of any non-viral transfection agent may be assessed and, eventually, improved. 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).

P814

Exploring low-cost non-viral mRNA delivery for retinal genetic diseases

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Gene therapy is an exciting therapeutic option to treat or delay the progression of several hereditary or chronic diseases. While clinical trials for viral vector-mediated gene therapy for numerous eye illnesses are still ongoing, there is consensus that research and development of other alternative therapies are needed. In this context, the non-viral delivery of *in vitro* transcribed (IVT) mRNA to cells offers many advantages, such as achieving high gene expression while eliminating the risk of genomic integration or the requirement to cross the nuclear barrier. In this study, we used human cell-based *in vitro* models, specifically (i) hiPSCs-RPE cells, which exhibit physiological characteristics of human RPE, and (ii) retinal organoids derived from hiPSCs, which mimic the cytoarchitecture of human retina, to explore the use of mRNA as a therapeutic tool for eye diseases. First, we used enhanced green fluorescence protein (eGFP) mRNA to compare the efficiency of mRNA over other therapies (AAVs and DNA strategies). Second, we tested the cellular uptake capacity and transfection efficiency in iPSCs-RPE cells and found that mRNA cell uptake has a peak at 6 h and GFP protein levels were still detected 30 days post-transfection in iPSCs-RPE. In 3D retinal organoids, several retinal cells were GFP-positive in deeper layers and in more developed organoids, photoreceptors were the main cells presenting GFP levels. After *in vitro* optimization, the eGFP mRNA delivery was examined in adult wild-type mice eyes after intraocular injections and high GFP levels were found within the majority of RPE cells and in the photoreceptors layer.

Taken together, we show that mRNA delivery exhibits highly efficient expression in 2D and 3D retinal cell models for up to a month. The obtained data highlight the potential of the IVT mRNA-based gene therapy in the retina and open new avenues for developing affordable gene therapies for retinal diseases.

P815

Pioneering lipid nanoparticle (LNP) development for efficient delivery of large pDNA in retinal gene therapy

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Retinitis pigmentosa (RP) represents a heterogeneous group of inherited retinal disorders affecting up to one in four thousand people worldwide. The disease is characterized by progressive degeneration of photoreceptor cells and retinal pigment epithelium at the back of the eye, leading to a diverse array of visual impairments and gradual vision loss. Although efforts have been made to mitigate symptoms and delay disease progression through interventions like low vision aids and protective eyewear, the treatment landscape for individuals diagnosed with RP remains devoid of targeted therapeutic options. In this study, we explore the potential of gene therapy as a solution

for RP by utilizing non-viral lipid nanoparticles (LNPs) as carriers for mediate to large pDNA (5 kB and 11kB), that exceed the packaging capacity of currently employed adeno-associated viral vectors (AAVs). Size, charge and encapsulation efficiency are evaluated by dynamic light scattering and PicoGreen assay. Uptake and transfection efficiency on 2D cell culture (ARPE19) is evaluated by flow cytometry and confocal microscopy, defining an optimized lipid based particle for pDNA with mediate length. For the larger pDNA cargo, flow cytometry, qPCR and western blot are employed to assess efficiency regarding uptake and translation level 24 hours after transfection. The best 5 kB pDNA-LNP composition exhibits a hydrodynamic diameter of 100 nm, neutral charge at physiological pH and 92% encapsulation efficiency. Transfection of this formulation yields 36.8% (± 4.6) of transfected cells after 24 hours. Furthermore, we demonstrate efficient encapsulation of large 11 kB pDNA in LNPs, with a hydrodynamic diameter of 130 nm and 96% encapsulation efficiency. This compelling evidence demonstrates the efficient encapsulation of large genes within LNPs. Via flowcytometry, an uptake of 97,2% was recorded, which is confirmed by confocal images. Ongoing research focuses on assessing transfection efficiency at the RNA and protein levels. Next steps include characterization of transfection efficiency and toxicity of these particles on advanced 3D organoid models and retinal explants. These preliminary finding highlight the potential of LNP-mediated gene therapy, not only for retinal disorders like RP, but also for other genetic disorders or applications involving large gene cargoes.

P816

The effect of electrostatic high pressure nebulization on the stability, activity and *ex vivo* distribution of ionic self-assembled nanomedicines

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Pressurized intraperitoneal aerosol chemotherapy (PIPAC) is applied to treat unresectable peritoneal metastasis (PM), an advanced, end-stage disease with a poor prognosis. Electrostatic precipitation of the aerosol (ePIPAC) is aimed at improving the intraperitoneal (IP) drug distribution and tumor penetration. Also, the combination of nanoparticles (NPs) as drug delivery vehicles and IP aerosolization as administration method has been proposed as a promising tool to treat PM. There is currently limited knowledge on how electrostatic precipitation (ePIPAC) and high pressure nebulization (PIPAC) affects the performance of electrostatically formed complexes. Therefore, the stability, *in vitro* activity and *ex vivo* distribution and tissue penetration of negatively charged cisPt-pArg-HA NPs and positively charged siRNA-RNAiMAX NPs was evaluated following PIPAC and ePIPAC. Additionally, a novel multidirectional Medspray® nozzle was developed and compared with the currently used Capnopen® nozzle. For both NP types, PIPAC and ePIPAC did not negatively influence the *in vitro* activity, although limited aggregation of siRNA-RNAiMAX NPs was observed following nebulization with the Capnopen®. Importantly, ePIPAC was linked to a more uniform distribution and higher tissue penetration of the NPs aerosolized by both nozzles, independent on the NPs charge. Finally, compared to the Capnopen®, an increased NP deposition was observed at the top of the *ex vivo* model following aerosolization with the Medspray® nozzle, which indicates that this device possesses great potential for IP drug delivery purposes.

P817

Production of *in vitro* transcribed mRNA using synthetic, enzymatically produced linear DNA

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The manufacture of high-quality, GMP grade DNA is a major bottleneck in the production of mRNA and viral vectors for use in gene therapy and vaccines. 4basebio has developed a proprietary, scalable, fully enzymatic synthesis process for the production of linear DNA constructs via our Trueprime™ amplification technology. The opDNA™ produced is devoid of any bacterial backbone and the manufacturing process circumvents cumbersome fermentation processes required for plasmid DNA. 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. Moreover, opDNA™ does not need to be enzymatically linearised prior to use in an IVT reaction, and long, continuous polyA tails of 120 bp in length can be coded into the DNA template.

Here, we compared the synthesis of *in vitro* transcribed (IVT) mRNA using opDNA™ versus linearised plasmid DNA across a panel of constructs ranging from 1.5kb to 9kb. Using opDNA™, we were able to achieve significantly higher yields as compared to linearised plasmid, and equivalent capping efficiency. Proinflammatory cytokine/chemokine levels in isolated primary, human PBMCs were comparable to mRNA produced from linearised plasmid, as was 3‘ heterogeniety of the transcripts. Luciferase and GFP expression both *in vitro* and *in vivo* was comparable to mRNA produced from linearised plasmid.

We have demonstrated that opDNA™ templates can be used for the production of IVT mRNA, which could greatly accelerate gene therapy therapeutic development. Moreover, the technology could overcome the difficulties associated with complex polyA tails for mRNA constructs, which are inherently difficult to synthesise via bacterial propagation systems.

P818

Nanoparticle-mediated delivery of CRISPR/Cas13 RNP for the knockdown of oncogenic proteins: A promising approach for cancer therapy

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The CRISPR/Cas13 System can specifically recognize and cleave ssRNA. Its capability as a biotechnological tool has been explored, including RNA knockdown and RNA editing. While most studies have been performed using DNA encoding for Cas13 systems, it is widely known that the delivery of RNPs (pre-formed protein-crRNA complexes) is a safe but more challenging possibility. Here, we studied the knockdown efficiency of Cas13b delivered as RNP into human cells, for the knockdown of GFP as a model target and kRas as an endogenous target. Furthermore, we used Cas13b to target survivin, a protein overexpressed in many types of cancer that plays a role in

promoting cell survival and preventing apoptosis. Thus, targeting survivin could have great therapeutic potential in the treatment of cancer.

We aim to use different nanovehicles to achieve a safe and efficient intracellular delivery of CRISPR/Cas13 and produce RNA-knockdown of the mentioned mRNAs.

P819

Unilamellar liposome encapsulation of the TFAMoplex DNA transfection agent

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Non-viral DNA transfection systems often suffer from limited stability in body fluids and aggregation. The encapsulation of DNA molecules into unilamellar liposomes could protect the DNA from aggregation, due to the reduced interaction with serum proteins and therefore increase its systemic circulation time. The aqueous core of a liposome further allows to co-deliver proteins beneficial for the intracellular protection of the DNA, such as human vaccinia-related kinase 1 (VRK1) against the cytoplasmic DNA defense system mediated by barrier-to-autointegration factor (BAF). However, the large dimensions of DNA molecules currently prevent its efficient encapsulation into neutral or negatively charged nanosized liposomes.

In this project, we developed a strategy to encapsulate our previously developed protein/DNA nanoparticles (TFAMoplex, based on human mitochondrial transcription factor A) into unilamellar liposomes consisting only of natural phospholipids and cholesterol. To increase the encapsulation efficiency, human annexins were fused to the TFAMoplex surface. The annexins were shown by confocal microscopy to recruit the TFAMoplexes onto a lipid film, in a reversible, calcium-dependent manner. This was followed by vesicle formation, in which the membrane-bound protein-DNA complexes were encapsulated. Subsequent extrusion and size exclusion purification resulted in unilamellar vesicles with an average diameter of 150 nm, as indicated by dynamic light scattering (DLS) and Cryo-Transmission Electron Microscopy (Cryo-TEM). The encapsulation of TFAMoplexes in the liposomes was verified by Nanoparticle Flow Cytometry (NanoFCA) and Flow Induced Dispersion Analysis (FIDA) using both fluorescently labelled DNA and proteins. To increase the uptake of the vesicles, the surface was coated with the uptake factor apolipoprotein E fused to annexin. This resulted in significantly increased vesicle uptake into HeLa cells as measured with confocal microscopy and flow cytometry.

In conclusion, we demonstrated that the TFAMoplex transfection system can be encapsulated into unilamellar vesicles by calcium-dependent membrane recruitment. However, it remains to be tested if the transfection efficiency of the system is maintained.

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P820

Adapting AIMer-based RNA editing technology for application in CNS

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We have previously shown that endogenous ADAR enzymes (adenosine deaminases acting on RNA) can be directed to perform sequence-specific RNA editing in liver through the use of chemically modified stereopure oligonucleotides called AIMers. Here, we describe the development of AIMer technology for application in the CNS. Initially using ubiquitous housekeeping transcripts as surrogate targets, we developed AIMers that directed substantial RNA editing in human iPSC-derived neurons and astrocytes. Following administration into transgenic mice that express human *ADAR1*, we demonstrate 25% to 65% RNA editing across the entire CNS, which was sustained to 16-weeks post-single injection. Administration of AIMers in non-human primates via intrathecal injection resulted in around 50% RNA editing in the spinal cord and up to 30% in the brain. To assess the ability to edit a clinically relevant target, we designed stereopure AIMers to edit the *MECP2* R168X mutation, the most frequent nonsense mutation that causes the rare neurodevelopmental disorder called Rett Syndrome (RTT). These AIMers are designed to convert the premature stop codon to tryptophan, promoting translation readthrough and protein restoration. In 293T cells, we demonstrate that MECP2 R168W protein localizes properly to the nucleus and interacts with expected co-regulatory proteins, suggesting functionality. In human and mouse neuronal lines, *MECP2* AIMers direct 30% to 90% RNA editing, respectively. With this level of in vitro editing, we restore full-length protein detectable by western and immunofluorescence assays. These preclinical investigations lay the foundation for the development of AIMers, with the potential to treat human diseases in neurology.

P821

A pseudotyped vesicle delivery system for genome editing in Cystic Fibrosis

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Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the Cystic Fibrosis Transmembrane Regulator (*CFTR*) gene. CRISPR-Cas9 editing tools have provided powerful and effective strategies to correct *CFTR* mutations in a wide variety cellular models. However future perspectives of in vivo applications are limited by the lack of efficient and safe delivery methods to target lungs, the most affected organs in CF patients. Promising delivery tools consist in viral derived vectors having efficient mechanisms of cellular entry and with tuneable tissue tropisms. This study exploits VEsiCas (Montagna *et al*, 2018) a delivery tool consisting in VSVG-enveloped vesicles which have been demonstrated to efficiently deliver CRISPR-Cas as ribonucleoprotein complexes (RNPs), thus limiting the off-target activity. Here we demonstrate that VEsiCas

efficiently delivers adenine base editors and a single guide RNA in multiple loci and in different cellular models (CFBE41o-, Huh7 and Caco2). We are currently evaluating the efficacy of our delivery tool in cell lines that we have produced carrying the most frequent *CFTR* nonsense mutations (R553X, R1162X and W1282X). Finally, in order to optimize vesicles' tropism towards lungs, we are testing various envelops with high targeting potential for airway epithelial cells and they will be tested in primary differentiated cells and in in vivo models.
With an engineered pseudotyped vesicles we aim to develop an efficient delivery method for genome editing technologies to take a step forward in CF treatment and potentially other lung diseases.

P822

Extracellular vesicles from mesenchymal stromal cells as potential angiogenic modulators through the delivery of RNAi-expressing minicircles

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Several conditions include excessive angiogenesis underlying the disease and thus blocking the vascular endothelial growth factor (VEGF) regulatory pathway can be a promising therapeutic approach. RNA interference is a possible avenue, and expression vectors encoding short-hairpin RNAs (shRNAs) can be used as an alternative to synthetic siRNAs. A promising vector system are minicircles (MC), which are safe non-viral vectors capable of high levels of transgene expression. Extracellular vesicles (EVs) secreted by mesenchymal stromal cells (MSC) (MSC-EV) retain important features of the parental cells, being candidates for cell-free therapies and promising gene delivery vehicles. In this context, we hypothesized that modification of MSC-EVs with shRNA-expressing MC that silence the expression VEGF (MC-shVEGF) and its receptor (MC-shVEGFR2) could represent a novel cell-free gene-based therapy to treat vascular overgrowth. Here, we established a platform for the scalable production of MSC-EV in serum-/xeno(geneic)-free (S/XF) conditions using human platelet lysate (hPL) supplements combining the DASbox® mini bioreactor system and ready-to-use, biodegradable microcarriers. By implementing an intermittent agitation regimen, we observed high cell adhesion rates (~80%) and achieved a ~30-fold cell expansion after 6-7 days. Afterwards, this platform allowed a continuous 3 day-production of MSC-EVs with an estimated productivity of $3.5x10^2$ particles.mL^{-1}/cell. Tangential flow filtration followed by anion-exchange chromatography allowed the isolation of MSC-EV that present the typical cup-shaped morphology, small size distribution and expressed EV-positive protein markers (CD63, CD9 and Syntenin). In addition, the scalable production and purification of the MC was accomplished, and their silencing potential was evaluated by RT-qPCR after cell transfection. RT-qPCR results showed that the MC-shVEGF induced a maximum knockdown of 78% in a human breast cancer cell line (MDA-MB-231) after 4 days, and the MC-shVEGFR2 led to a knockdown of 45%, 2 days after transfecting umbilical vein endothelial cells. Finally, MSC-EVs loading with the MC that targets GFP was tested by passive incubation, microporation, sonoporation and using a transfection reagent. The loading efficiency was determined using Rho-labeled MC and the functional EV-MC delivery was evaluated by flow cytometry after transfecting GFP-expressing HEK293 cells (HEK-GFP+). The results were similar for the different methods studied showing that MSC-EVs loading efficiencies were low (<7%), with

no significant decrease in the median fluorescent intensity of HEK-GFP+, 48h after EV-MC delivery. Overall, in this work we successfully developed a scalable S/XF microcarrier-based stirred culture system for the robust production and isolation of MSC-EV. Additionally, this study provides important insights towards the implementation of an MC-derived RNAi-based system that targets angiogenesis and the potential use of MSC-EVs as gene delivery vehicles, suggesting that several challenges must be addressed to broadly establish EVs as DNA delivery vehicles.

P823

Pre-formation loading of extracellular vesicles with exogenous molecules by photoporation

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While initially thought that the function of extracellular vesicles (EVs) was merely waste disposal from cells, their crucial role in intercellular communication has become more clear. The notion that EVs have the capability to transfer bioactive cargo from one cell to another has drawn the attention of the drug delivery field. Interest in repurposing EVs for drug delivery was further sparked by their good biocompatibility and targeting capabilities. The use of EVs as drug delivery carriers is, at the moment, hampered by the difficulty of loading EVs with exogenous compounds in a manner that does not interfere with their normal functioning and surface properties. We therefore propose vapour nanobubble (VNB)-mediated photoporation as pre-formation EV loading method. This technique uses photothermal nanoparticles and pulsed laser light to create transient pores into the plasma membrane through which cargo molecules are able to passively diffuse directly into the cell's cytosol. We hypothesized that by first delivering cargo molecules into the cytosol of parent cells, they would subsequently be internalized into the lumen of EVs during biogenesis.

As EV producer cell line, we used HEK293T cells transiently expressing the gag-EGFP fusion proteins, which allow for easy evaluation of EVs. To demonstrate loading of EGFP EVs, we used two fluorescently labelled cargo molecules: dextran macromolecules, which do not specifically interact with intracellular structures, and anti-EGFP nanobodies, targeted toward EGFP-labelled EVs. Both compounds were first delivered into the cytosol of *in vitro*-cultured cells using VNB-mediated photoporation. After purification, EV loading efficiency was assessed using ExoView®, which allows for single-EV analysis and quantification of EV loading. Finally, impact of the laser treatment on typical EV characteristics like concentration, size, zeta potential and expression of tetraspanins was assessed.

We demonstrated that a fraction of the delivered dextrans or nanobodies was passed from the parent cell's cytosol into the formed EVs. Importantly, nanobody loading was clearly superior to the loading of dextran macromolecules, reaching ~20% of EGFP EVs containing nanobodies, which amounts to ~9% of the total EV population. Importantly, we established that laser-induced VNBs have minimal impact on the EV characteristics.

In conclusion, this work demonstrates the feasibility of VNB-mediated photoporation for pre-formation loading of EVs, while minimally impacting EV characteristics. In future work it will be of interest to evaluate EV loading with nucleic acids as well.

P824

Characterization of enhanced lipid nanoparticle delivery after photodisruption of the inner limiting membrane

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Intravitreal injection is an interesting route of administration to target the retina given its low invasive and easy to perform character. However, after injection into the vitreous, the inner limiting membrane (ILM) represents a major hurdle for therapeutics to efficiently reach retinal target cells. Aware of this barrier, we explore indocyanine green (ICG) mediated photodisruption to perforate the ILM in a local and controlled manner. In this concept, the photosensitizer ICG is delivered to the ILM followed by treatment with high intensity laser pulses generating vapor nanobubbles. Upon their collapse, mechanical effects are able to disrupt the ILM paving the way for therapeutics to enter the retina. Given the increasing interest in mRNA therapy to treat retinal diseases, the goal of this *ex vivo* study is to characterize the enhancement of mRNA lipid nanoparticle (LNP) delivery after ILM photodisruption. In this regard, luciferase encoding mRNA was encapsulated into LNPs composed with the ionizable lipid C12 200 via microfluidic mixing. Based on Dynamic Light Scattering, LNPs were characterized by a size of approximately 100nm and a neutral charge. To disrupt the ILM, ICG was applied on top of bovine retinal explants followed by pulsed laser treatment (7ns, 800nm). After photodisruption, explants were cultured and transfected with LNPs for 24h. Finally, cryosections were prepared followed by immunostaining with collagen IV antibodies to investigate ILM integrity as well as retinal entry of LNPs with confocal microscopy. As expected, LNPs were blocked at the intact ILM since limited retinal penetration was observed in untreated samples. On the contrary, ILM damage provoked by the photodisruption treatment resulted in substantial penetration of LNPs into the inner retinal layers. To investigate whether this enhanced penetration furthermore boosted mRNA expression, the bioluminescent signal of the explants - resulting from luciferase expression - was measured after 24h of transfection by use of the IVIS equipment. Strikingly, the improved LNP entry indeed enhanced the efficacy since a 4-fold increase in luciferase expression was obtained compared to explants without photodisruption treatment. To conclude, our results demonstrate that the delivery of LNPs can be substantially enhanced by ILM photodisruption. Moreover, the improved retinal LNP entry led to a significant increase in mRNA expression. Considering these promising results, future experiments will focus on finding the optimal efficacy safety balance by proper toxicity analysis of the treatment as well as exploring realistic target cells for LNP delivery to the retina.

P825

Extracellular vesicles-mediated delivery of CRISPR/Cas9 systems to treat Machado-Joseph Disease/Spinocerebellar Ataxia Type 3

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Machado-Joseph Disease (MJD), also known as Spinocerebellar Ataxia Type 3 (SCA3), is a neurodegenerative dominantly inherited disorder caused by an abnormal over-repetition of a CAG tract within the ataxin-3 gene. To correct these defects, CRISPR/Cas9 systems allow precise gene editing of disease-causing mutations. However, long-term expression of CRISPR/Cas9 in target tissues leads to an increased in off-target activity and immunogenicity. To counteract this problem, transient delivery of the CRISPR machinery is preferable since it allows potent on-target editing followed by rapid ribonucleoprotein (RNP) turnover. To that aim, extracellular vesicles (EVs) were shown to be promising vehicles to transiently deliver CRISPR/Cas9 tools.

In this work, the goal was to generate an EV-based delivery platform for CRISPR/Cas9 systems to knock out ataxin-3 and ameliorate MJD hallmarks.

To encapsulate the CRISPR machinery in EVs, an EV-packaging signal (EVPack) was associated with SpCas9 nuclease. The nuclease activity and EV-packaging efficiency of the recombinant EVpack-SpCas9 was evaluated by Western Blot, RT-qPCR and bioluminescence. To test the efficacy of the system, EVs enriched with CRISPR/Cas9 RNPs were incubated in recipient cells and ataxin-3 knockout was assessed at the DNA and protein level. Moreover, aiming to improve cell uptake and cargo delivery VSV-G was engineered on EVs surface. The efficacy of this delivery toolbox was tested *in vitro* and *in vivo*. Finally, CRISPR EVs were intracranially injected in the cerebella of the YAC 84.2 MJD mouse model.

Western blot and immunocytochemistry results showed that the EVPack signal did not impair SpCas9 nuclease activity. Moreover, EVPack-SpCas9 modified cells significantly enriched SpCas9 protein and sgRNA in EVs. EVs enriched with CRISPR/Cas9 RNPs knocked out ataxin-3 expression in recipient cells. VSV-G engineered EVs improved delivery of CRISPR/Cas9 tools to recipient cells. Remarkably, CRISPR EVs were able to mediate genome editing in the YAC 84.2 MJD mouse model.

These results provide evidence that an EV-based transient delivery of CRISPR/Cas9 tools partially knockout ATXN3 expression and possibly mitigate the deleterious effects of CRISPR/Cas9 long-term expression in eukaryotic systems.

P826

Modified small interfering RNA for progerin downregulation in fibroblasts from patients with Hutchinson-Gilford progeria syndrome

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Hutchinson-Gilford Progeria Syndrome (HGPS) is an exceedingly rare genetic disorder that arises from a mutation occurring in the *LMNA* gene, responsible for encoding nuclear envelope filaments known as lamins A and C. Specifically, this mutation generates a novel splicing site within exon 11 of the lamin A coding sequence, while lamin C coding sequence remain unaffected. Consequently, the incorrect splicing gives rise to a lamin A protein that lacks a portion of the sequence encoded by exon 11. Deleted segment is crucial for the accurate posttranslational maturation of progerin, a mutated variant of lamin A. Due to improper maturation, progerin becomes permanently anchored at the inner nuclear membrane through a farnesyl group attached to its C-terminus. The accumulation of progerin disrupts the nuclear lamina, triggering significant alterations in cellular processes such as chromatin organization, transcriptional activity, and signaling pathways. Together, these cells abnormalities induce severe physiological changes within the organism, particularly affecting the cardiovascular system, ultimately resulting in mortality at an age of approximately 14 years. Presently, lonafarnib represents the sole therapeutic option available for HGPS patients. While this drug can extend patients' lifespans, it does not reverse the manifestations of the syndrome.

In our study, the primary objective was to identify small interfering RNAs (siRNAs) that exhibit selective recognition of progerin mRNA, subsequently promoting its degradation *via* RNA interference. Furthermore, we aimed to enhance the stability and resistance to degradation of siRNAs by incorporating modified nucleotides into their sequence. Concurrently, we aimed to ensure that the selected siRNA sequences do not impact the expression level of unmutated lamin A, regarding the fact of the importance of a high lamin A/progerin ratio for a positive therapeutic outcome.

Initially, we designed various siRNA sequences and assessed their efficacy in HeLa cells overexpressing either GFP-lamin A or GFP-progerin. The most effective siRNAs were then evaluated in human fibroblasts obtained from patients with progeria syndrome. To enhance the stability of the oligonucleotides, we incorporated nucleotides with fluoridated ribose, which resulted in increased stability, as confirmed by a stability assay conducted in mouse serum. Our findings demonstrated that the sequential administration of the designed siRNAs led to a substantial reduction in progerin levels in the fibroblasts derived from patients. Importantly, this reduction was achieved without affecting the levels of lamin A or lamin C, as western blot analysis had shown. Both the modified and non-modified siRNAs exhibited high efficiency and comparable specificity, indicating their suitability for further *in vivo* examination.

Altogether, tested oligonucleotides present a promising approach for inhibiting progerin synthesis and accumulation in patients' cells. This opens up avenues for potential gene therapy using either double-stranded siRNA conjugated with a carrier or one-stranded antisense oligonucleotides, which eliminate the need for conjugates in the delivery process. Additionally, an alternative strategy involves the overexpression of the designed sequence as miRNA or shRNA following viral transduction. Presented results offer potential routes for therapeutic development and warrant further exploration in the context of progeria syndrome treatment.

P827

Development of In Vitro Transcribed mRNA Therapeutics for Cystic Fibrosis

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Cystic fibrosis (CF) is an autosomal recessive disorder caused by a mutation in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. The dysfunctional CFTR protein leads to production of a thick sticky mucus, chronic bacterial infection and loss of lung function. Modulator drugs provide effective therapies for some CFTR genotypes but approximately 20% of patients remain untreatable. For this group we propose to develop in vitro transcribed CFTR messenger RNA (mRNA) as a novel therapy. We have developed an epithelial cell targeting nanoparticle to improve mRNA delivery comprising targeting peptides and lipids. The optimal design for transfection of 16HBE epithelial cells comprised the cationic lipid, ditetradecyl trimethyl ammonium propane (DTDTMA), the neutral helper lipid, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and peptide Y (K_{16}GACYGLPHKFCG) with luciferase mRNA which led to high transfection efficiency. The second part of this project is to optimize the in vitro transcription process and mRNA structural elements to produce synthetic mRNA with high translation efficiency, stability, and low inflammatory potential. The in vitro transcription protocol was optimized with the RNA cap structure analog, 3'-O-Me-m^7G(5')ppp(5')G, added during in vitro transcription to allow co-transcriptional capping. Unmodified uridine-5'-triphosphates (UTPs) were replaced by modified UTPs, N1-methylpseudouridine-5'-triphosphate. Double strand RNA contaminants were removed by cellulose-base purification. Various 5' untranslated regions (UTR) and 3'UTRs were cloned into the plasmid. According to this optimised protocol, the luciferase mRNA produced transfected 16HBE and CFBE cells with similar efficiency to commercially available luciferase mRNA. Taken together, this work provides the basis for the development of targeted gene therapy for cystic fibrosis that precisely delivers mRNA to epithelial cells in the lung.

P828

Preparation and Evaluation of Novel Cationic Niosomes for Efficient Gene Delivery to Breast Cancer Cells

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Gene therapy is an intracellular delivery of genomic materials into specific cells for the treatment of genetic and acquired disorders. In order to have an efficient therapeutic effect, nucleic acids should be delivered stably into the cells. Delivery systems are needed for efficient delivery of genes. In this study we have developed nano-sized niosomes and niopolyplexes as non-viral DNA delivery systems, for LV-RFP plasmid DNA (pDNA) as a model gene, and evaluated their efficacy and safety profile for gene delivery to breast cancer cells. Polyplexes were prepared by complexing branched polyethylenimine (BPEI) with pDNA at a ratio of 1:1 (w:w) and complexed

with niosomes to form niopolyplexes. Gene delivery efficiency, toxicity, stability and hemolytic activity of niopolyplexes were evaluated for either ER (+), PR (+) and HER2 (-) MCF-7 human breast cancer cells or MCF-12A human breast cell line. Transfection of MCF-7 breast cancer cells was achieved by prepared cationic niosomes which had a particle size of 51.89 nm and a zeta potential value of +28.3 mV. Although niopolyplexes were toxic to MCF-7 breast cancer cells, they were non-toxic to human breast cell line MCF-12A. As a result, nanosized niopolyplexes were prepared as stable and efficient delivery systems for MCF-7 breast cancer cells, which have the potential to be used as suitable delivery systems for development of gene therapy medicinal products after preclinical and clinical studies.

P829

Investigation of RNA-targeting CRISPR-dCas13 system to restore granulin expression in Frontotemporal Dementia

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Mutations in granulin (*GRN*) gene are the second major genetic cause of frontotemporal lobar degeneration (FTD) and appear all throughout the gene. A non-coding mutation in the intron 7 of *GRN*, known as c.709-1G>A, has been reported to be specific of Basque population and some other rare cases in the world. However, the molecular mechanism by which this intronic variant causes FTD is unknown. In this work, we establish the effect of *GRN* c.709-1G>A mutation *in silico*, *in vitro* and in patient samples. We observe that the mutation leads to an aberrant splicing pattern, which causes *GRN* mRNA degradation and progranulin haploinsufficiency. Next, we investigate whether the mutation is suitable for splicing modulation approaches using RNA-targeting CRISPR-dCas13 system. Similar to already approved splice-switching antisense oligonucleotides, this system binds to specific sequences in a target pre-mRNA, sterically blocks access of RNA-binding proteins and therefore modulates RNA-processing pattern. We aim to investigate the use of CRISPR-dCas13 system to develop a personalized therapeutic approach that could rescue granulin expression and therefore FTD phenotype.

P830

Nucleic Acid Delivery Using Nanoparticle Formulations to Treat Cystic Fibrosis

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Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. The dysfunctional CFTR protein leads to thickened mucus, persistent bacterial infections, and reduced lung function. Modulators are

effective treatments for many *CFTR* genotypes, but approximately 20% of patients remain untreatable. Therefore, we are developing an alternative therapy to be delivered by receptor-targeted nanocomplexes (RTNs). We aim to improve nucleic acid delivery to lung cells using a novel targeted nanoparticle formulation. The first aim was to explore the cellular pathway involved in nanoparticle transfection. The second aim was to determine whether delivery exacerbates inflammation, already present in the CF lung, by measuring cytokines. Plasmid DNA (pDNA)/mRNA, encoding GFP or luciferase, was packaged into RTNs, comprising a liposome formulation of ditetradecyl trimethyl ammonium propane (DTDTMA), dioleoyl L-α phosphatidyl ethanolamine (DOPE) at a 1:1 molar ratio, combined with a peptide, K_{16}-GA-CSERSMNFCG. 16HBE14o- and CFBE F508del cells were transfected with RTNs, and IL-6 protein secretion measured by ELISA. Air-liquid interface (ALI) cultures, a cell culture model more closely resembling the cellular environment of the respiratory tract, is also being investigated. Nuclear uptake and gene expression were evaluated by confocal microscopy, flow cytometry and luciferase assays. Endosomal escape was assessed by adding chloroquine, an endosomal buffer, alongside transfection. Cellular uptake and gene expression, measured by Cy-5 labelled GFP pDNA and flow cytometry at 24 hours post-transfection, was higher in CFBE cells (99.1% and 37.1% respectively) compared to 16HBE14o- cells (63.9% and 21.7% respectively). GFP expression for a GFP mRNA transfection, assessed by flow cytometry, was higher in CFBE cells (69.6%) compared to 16HBE14o- (16.0%) cells. The microscopy demonstrated that many RTNs were perinuclear 48 hours post-transfection in 16HBE14o- cells, suggesting that nuclear uptake is limiting GFP expression. Chloroquine reduced the RTN transfection efficiency in 16HBE14o- cells, but improved efficiency when RTNs lacked the lipid component. This effect will also be investigated in CFBE cells. Taken together, this work suggests that the major obstacles to transfection were cellular and nuclear uptake in 16HBE14o- cells, and nuclear import, transcription, nuclear export and/or translation in CFBE cells. Nanoparticle transfections did not significantly increase IL-6 levels in CFBE cells; other cytokines are currently being explored in CFBE cells and ALI cultures. There was no evidence, from IL-6 measurements, of an inflammatory response to RTN transfection in CFBE cells. CFBE cells and ALI cultures, which more closely mirror the lung environment compared to cell lines, will be used for future experiments. *In vivo* work will further assess the safety and efficiency of nanoparticle delivery. A gene editing strategy will be developed to correct *CFTR* mutations using the RTN formulation described in this study.

P831

Targeted delivery and long-term stability of the Hermes nanoparticle platform *in vitro* and *in vivo*

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4basebio specialises in the design, manufacture and supply of application specific synthetic DNA or mRNA and, can encapsulate these payloads in the Hermes™ non-viral delivery system. 4basebio's scalable, fully enzymatic linear DNA synthesis process via our Trueprime™ amplification technology enables production of hp, op, os or oeDNA™ without a bacterial backbone or an antibiotic resistance marker. Our 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.

The Hermes™ non-viral delivery system is a modular nanoparticle vector consisting of lipids and targeting ligands that can encapsulate a range of payloads including mRNA, DNA, siRNA, miRNA

and Cas9 RNP complexes. The targeting ligand can be customised to target cells or tissues, depending on the application, and works synergistically with the lipids to achieve *in vitro* transfection efficiency <90% following mRNA delivery in a range of cell types, including primary cells, and <70% following delivery of 4basebio's linear hpDNA. This relationship between the lipid and targeting ligand facilitates a reduced lipid content compared to conventional lipid nanoparticles (LNPs), improving *in vitro* toxicity. *In vivo* expression of luciferase mRNA delivered with Hermes is equivalent to that of conventional LNPs following intramuscular delivery and, by manipulating the ligand-lipid relationship, targeting to non-hepatic organs has been achieved following intravenous delivery.

Finally, the incorporation of the targeting ligand offers enhanced long-term stability at 4°C compared to conventional LNPs, overcoming cold chain requirements. Hermes nanoparticles encapsulating an mRNA cargo maintained a uniform size and PDI for one year when stored at either +4°C or -80°C degrees, without a drop in transfection efficiency in HEK293T cells. Combined with the *in vivo* transfection efficiency, this makes the Hermes system ideally suited for vaccine applications.

P832

Structure-performance study of micelles based on oppositely charged block copolymer for application in gene delivery

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The COVID-19 mRNA vaccines represent a milestone in developing non-viral gene carriers, and their success highlights the crucial need for continued research in this field to address further applications. However, there are several hurdles to overcome. Many of which can be addressed with cationic polymer-based gene delivery systems due to their durability, reproducibility, large production quantities, versatile composition, and architecture, in particular for pDNA. However, systems based on cationic polymers often face the dilemma of balancing toxicity and efficiency. Introducing anionic, hydrophilic, or "stealthy" functionalities represents a promising approach to overcoming this dilemma in gene delivery. To study the structure-performance relationship, polymers with the same composition but varying arrangements of polymer units were synthesized: (i) differently charged diblock terpolymers comprising hydrophobic poly(n-butyl acrylate), a copolymer segment made of hydrophilic 4-acryloylmorpholine (NAM), and either the cationic 3-guanidine propyl acrylamide (GPAm) or the 2-carboxyethyl acrylamide (CEAm), which is negatively charged at neutral conditions, (ii) triblock quaterpolymers composing hydrophobic poly(n-butyl acrylate) unit, followed by a copolymer segment of NAM and CEAm and lastly a GPAm block, (iii) triblock quaterpolymers with the same composition units, but with a reverse arrangement of the cationic and anionic block. The oppositely charged sets of diblocks (i) are co-assembled in different ratios to form mixed micelles and the triblocks (ii) and (iii) were assembled to form triblock micelles.

Tests were initially carried out with mixed micelles to determine the ideal positive-to-negative charge ratio for achieving maximum transfection efficiency and cell viability. This approach did not require additional polymer synthesis and can be performed with minimal resource expenses. For efficient complexation of genetic material, a defined proportion of cationic diblock copolymer

could be determined. Moreover, an optimal mixing ratio of positive-to-negative charge was successfully identified to avoid strong serum interaction and create suitable membrane interaction for effective transfection by retaining biocompatibility. To further understand the relationship between structure and performance, triblock quaterpolymers were synthesized with comparable positive-to-negative charge ratios as the most effective mixed micelles but variations in the arrangement of the building blocks. Indeed, when the triple blocks reached a certain charge ratio, the influence of serum proteins increased, leading to a decrease in transfection efficiency. However, in the case of the reverse triblock, cell viability is enhanced. In conclusion, it is worth noting that triblock micelles featuring a negatively charged copolymer segment in the outer shell area were less impacted by serum interaction and were able to effectively interact with cell membranes, resulting in successful transfection and high viability. Overall, the findings of the study on mixed micelles and triblock micelles emphasized the significant influence of the negatively charged copolymer segment and their defined molar arrangement provided by polymers. The results highlighted the impressive potential of integrating negatively charged units into the cationic polymer structure to enhance its effectiveness in terms of transfection efficiency and viability.

P833

siRNA therapy against lung cancer via polyallylamine-oleic acid delivery system

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The use of small interfering RNA (siRNA) to silence genes has revolutionized potential therapies for genetic disorders, including lung cancer, the most common and lethal cancer worldwide. This work investigates a novel approach to siRNA therapy, targeting CD47 transmembrane protein, which is overexpressed in tumors and act as a "don't eat me" signal to macrophages via SIRPα recognition. Blocking CD47-SIRPα interaction will promote phagocytosis in lung tumors. Despite the potential of CD47 siRNA therapy, effective delivery remains a major challenge, as most carriers accumulate minimally in tumors, predominantly reaching the liver. To address this problem, we present a novel siRNA delivery system that integrates polymers and lipids, specifically polyallylamine grafted with oleic acid in various molar ratios. Our system improves the balance between cell viability and siRNA transfection *in vitro*, evidenced by decreased CD47 mRNA and protein levels after oleic acid grafting at 6 and 14% degree of substitution. The biodistribution study in wild-type models using a radio-labelled siRNA showed increased accumulation in the lungs, contrary to free siRNA, which was rapidly cleared through the bladder after intravenous administration. Furthermore, after intratumoral administration in a tumor xenograft model with A549 cells, complexed siRNA showed prolonged tumor retention compared to free siRNA. Taken together, our findings highlight the promise of our innovative polyallylamine-oleic acid delivery system for siRNA-based lung cancer therapy, which could lead to significant advances in therapeutic outcomes.

P834

Efficient *in vitro* and *in vivo* mRNA and CRISPR delivery with lipid nanoparticles

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The RNA-guided programmable gene editing technology CRISPR-Cas9 has shed promising light for curing genetic disorders. Despite significant progresses in gene editing technologies have been achieved over the last decade, efficient and specific delivery of the CRISPR therapeutic components into target cells and tissues remains a major hurdle in therapeutic applications. Lipid nanoparticles (LNP) has evolved as a robust, efficient, and safe strategy for *in vitro* and *in vivo* delivery of mRNA, owing to several advantages such as high encapsulation efficiency, stability, protected from degradation, and easy to be functionalized. To evaluate and further advance LNP-based mRNA and CRISPR therapy, we systematically evaluate the LNP delivery efficiency using *in vitro* cultured cells, *ex vivo* cultured tissues, and *in vivo*.

Homogenous nanoparticle size distribution (100-200 nm) was achieved using staggered herringbone microfluidics mixing of ionisable lipids, helper lipids, cholesterol, and PEGylated lipids (ratio of 50:10:39:1). To quantify the delivery efficiency, we encapsulate a cargo mRNA encoding the enhanced green fluorescent protein (EGFP) in LNPs. Our preliminary results showed that LNP can efficiently deliver the *EGFP* mRNA into several well-established cell lines, primary cells (human fibroblasts, human PBMC lymphocytes, human mesenchymal stem cells, mouse Schwann cells, and mouse primary cortical neurons), iPSC-derived astrocytes and microglia, and hard-to-transfect cells (human endothelial cells), except myeloid cells, CD34+ HSCs, and iPSC-derived motor and cortical neurons. Promising mRNA delivery with LNPs was also observed in *ex vivo* mouse brain slice culture and *in vivo* subretinal injection. Furthermore, efficient NHEJ and HDR gene editing by LNP-mediated delivery of Cas9 mRNA, synthetic gRNA, with/without a single-stranded oligodeoxynucleotides (ssODN) template was also achieved in cultured cells. Overall, while future improvements are needed, our results showed that LNP is a promising delivery strategy for advancing CRISPR/Cas9-mediated gene editing and RNA therapeutics.

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Bactofection-mediated therapy with Deoxyribonuclease 1-Like 3 in chemically induced ulcerative colitis

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Extracellular DNA is involved in the pathogenesis of inflammatory diseases making it a promising therapeutic target. The strict localization of ulcerative colitis within the intestines could be explained by the low endogenous DNase activity in the colon. Bacteria-mediated gene transfer is an innovative therapeutic approach suitable especially for diseases affecting microflora-associated mucosae. In this study, we investigated whether preventive bactofection using *S. typhimurium SL7207* carrying murine Deoxyribonuclease 1-Like 3 (DNASE1L3) plasmid alleviates the symptoms in a chemically induced murine model of ulcerative colitis. *S. typhimurium* SL7207 was heat-transformed with PCMV6-Kan/Neo Mammalian Expression Vector carrying the murine DNase1L3 gene insert under the CMV promotor to yield *S.typhimurium* pDNase1L3 and grown in selective medium. Adult C57BL/6 mice were orally gavaged with >10^7CFU of *S. typhimurium* pDNase1L3 or an empty vector a day prior to the start of colitis, induced with 2% dextran sodium sulphate dissolved in tap water. Body weight and stool consistency were monitored for 7 days. Upon sacrifice, colon length was recorded and plasma extracellular DNA as well as serum nuclease activity were measured. Colitis was induced successfully as proved by all established signs of the disease. Treatment with DNase1L3 did not affect stool consistency, bleeding or the colon length. Bodyweight loss was even higher in the treated mice. No differences between the groups were found in plasma extracellular DNA or nuclease activity. Bactofection-mediated DNase1L3 gene transfer does not improve murine colitis. This could be due to technical limitations, but also due to fragmentation instead of elimination of extracellular DNA in vivo. Further studies focused on the treatment of sterile inflammation should test the effects of DNase1L3 in combination with DNase1.

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In vitro and in vivo plasmid-mediated gene therapy targeting extracellular DNA using deoxyribonuclease I

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Extracellular DNA (ecDNA) is not only an important marker of diseases but also a potential therapeutic target. EcDNA is released upon cell death from cells into circulation. In conditions such as sepsis, liver failure and inflammatory bowel diseases concentration of ecDNA were shown to be increased in animal models as well as in human patients in comparison to healthy controls. Additionally, ecDNA has proinflammatory properties which is especially true for that of mitochondrial origin which is a known damage-associated molecular pattern. EcDNA can be

removed from circulation by deoxyribonucleases (DNases). Cleavage of ecDNA could prevent sterile inflammation caused by ecDNA. Exogenous DNase I treatment improved survival and functional parameters, but this needs to be applied at least twice daily. We hypothesized that administration of plasmid vector carrying DNase I gene would increase DNase activity in plasma. In vitro experiments targeted bacterial heat shock transformation of competent *E. coli* BL21(DE3) with vector pCMV6-Kan/Neo-DNaseI carrying mouse DNase I or pcDNA3 (empty vector) and pcDNA3-GFP as controls. Bacteria were then grown on selective medium. HEK293 cell were transfected with the plasmid pCMV6-Kan/Neo-DNaseI to assess the expression of DNase I in eukaryotic cells. In vivo experiments in mice included intramuscular administration, combination with electroporation (two 100V pulses 10 ms) and hydrodynamic administration of 1 ug, 10 ug and 50 ug plasmid vector pCMV6-Kan/Neo-DNaseI. Finally, mice pretreated with hydrodynamic injection of pCMV6-Kan/Neo-DNaseI underwent surgical model of sepsis. DNase activity was measured in plasma using single-radial enzyme-diffusion assay. The transformation of *E. coli* with plasmid carrying mouse DNase I was successful, yielding colonies on the selection media. However, *E. coli* was slow growing with low viability. This was most likely caused by the large plasmid or potentially expression of DNase I due to leaky promotor. Next, transfection of HEK293 cells with plasmid carrying mouse DNase I was successful with cells expressing DNase I and secreting it into medium. DNase activity was increased in cell medium and cell lysates after 24, 48 and 72 h. In subsequent in vivo experiments, however, no difference in DNase activity in plasma was observed in up to 96 hours post intramuscular administration of plasmid nor when intramuscular administration was accompanied by electroporation. The hydrodynamic route of administration increased DNase activity in plasma of two mice, which led us to choose this administration route for following experiments. DNase activity in sepsis increased in time and was higher after 24 h. However, hydrodynamic administration of plasmid with DNase I did not lead to higher DNase activity in sepsis in comparison to sepsis control without plasmid. To summarize, plasmid-based gene delivery of DNase I represents a promising strategy for diseases associated with high concentrations of ecDNA. By facilitating the breakdown of extracellular DNA DNases could prevent the sterile inflammation. Our next experiments will focus on assessment of the increase of DNase activity in tissues as well as fragmentation of ecDNA.

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Chemical optimization of miR-146a mimic for the treatment of Graft versus Host disease

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Mice transplanted with miR-146a-/- T cells show more aggressive GvHD, and pretreatment of T cells a commercial miR-146a mimic (nucleofection), lower GvHD mortality and severity can be achieved. Here, we generated cholesterol-conjugatedmiR-146a mimics spanning chemical scaffolds from miRNA-like to siRNA-like in order to identify a clinically relevant scaffold for GvHD therapy. We tested the impact of (1) the length of the sense strand, and (2) the complementarity of the sense strand on miRNA efficacy in reporter and in functional assays. We first cloned a fully complementary target site 4x in tandem into the 3'UTR of a dual luciferase reporter system to test an siRNA-like effect. In this fully complementary target setting an siRNA-like structure led to less efficient silencing. Then, we used an in vitro model of GvHD, mixed lymphocyte reactions, to compare T cell inhibition functionality of miRNA mimics. Here we saw that both a shorter or a fully complementary sense strand significantly enhanced the T cell inhibitory effect. A further enhancement could be achieved by increasing the number phosphorotioate modifications in the antisense strand. We achieved the most potent T cell inhibition (up to 80 %, IC 50 216 nM) using

a chemical structure intermediate structure between miRNA and siRNA patterns. Collectively, we demonstrate that (1) some elements of siRNA chemical design may augment miRNA mimic efficacy in a functional setting, and (2) antisense strand metabolism is rate-limiting for miRNA functionality.

P838

Aptamers as potential therapeutics for molecular targets in chronic heart failure

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Myocardial infarction (MI) is among leading causes of death within global population. A common complication of MI, which develops in approximately 30% of patients, is heart failure (HF). HF with reduced ejection fraction (HFrEF) makes up 50% of all the HF cases worldwide. In HFrEF the left ventricular is enlarged with ejection fraction below 40%, leading to lack of oxygenized blood. Ca2+ transport through SERCA2a channel, which regulates heart contractility, is disrupted in HFrEF due to the overinhibition of SERCA2a by Phospholamban protein (PLN). Several studies confirmed increased levels of unphosphorylated PLN in mice and in human patients with aberrant Ca2+ transport.

Aptamers are easy to produce and customize. They efficiently disrupt protein-protein interactions by capturing the target protein. Inhibition of PLN-SERCA2a interaction by aptamers would facilitate Ca2+ uptake and consequently improve contractility of heart damaged with HFrEF. This study aims to test ssDNA and ssRNA aptamers as inhibitors for Phospholamban in HFrEF *in vivo*. Aptamers are produced and selected by the SELEX-process. Inhibition of PLN-SERCA2a by aptamers *in vitro* is studied on iPSC-derived cardiomyocytes and murine primary cardiomyocytes with and without PLNR14Δ/Δ mutation. Therapeutic effects of aptamers *in vivo* are studied on PLNR14Δ/Δ mouse model and on a new HFrEF model developed by inducing hypertension with angiotensin II minipumps in mice for 8 weeks. Cardiac functions will be monitored by ultrasound and MRI non-invasively. Histopathology methods are used to analyze HFrEF characteristics in myocardial sections.

The challenges and early results of SELEX and *in vitro* studies of aptamers will be discussed in detail in the poster. Preliminary examination of murine heart sections with HF induced by AngII immunohistochemically stained with PLN antibodies demonstrated a decline of PLN in comparison with control sections and accumulation of PLN around the MI injury. It suggests that heart is compensating for reduced contractility in these areas making aptamers attractive novel therapeutic molecules to improve cardiac function.

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Interleukin-12 antibiotic resistance gene-free plasmids for gene electrotransfer-mediated cancer immunotherapy

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Gene electrotransfer (GET) of plasmids encoding the cytokine interleukin 12 (IL -12) is an attractive gene therapy approach for cancer immunotherapy. In our department, we have been testing this approach over the last few decades, either as monotherapy or in combination with radiotherapy, or as an adjuvant to vaccination. Finally, in 2021, we initiated the first-in-human clinical trial of IL -12 GET in Europe for the treatment of cutaneous basal cell carcinoma tumors in the head and neck region (ClinicalTrials NCT05077033). To enter clinical trials in Europe, plasmids must meet EMA regulatory requirements that inter alia recommend against the use of antibiotics in the manufacture of clinical-grade plasmids. Here we present the preparation and testing of antibiotic resistance gene-free plasmids encoding mouse, canine, and human IL-12. The mouse ortholog was prepared to facilitate preclinical evaluation in mouse tumor models, the canine ortholog for cancer treatment of client-owned dogs, and the human ortholog for translation into human clinical trials. Our aim was to evaluate the maintenance of these plasmids in bacterial culture and to test the transfection efficiency after GET to melanoma cell lines.

Plasmids encoding mouse (pORF-mIL-12- ORT), canine (pORF-caIL-12- ORT), and human IL -12 (pORF-hIL-12- ORT) were prepared using the antibiotic-free selection strategy called Operator Repressor Titration (ORT®, Cobra Biologics) and all have the same backbone without antibiotic resistance gene. A commercially available plasmid encoding a mouse IL -12 (pORF-mIL-12 (p40p35), Invivogen) with an ampicillin resistance gene in the backbone was used as a control. Plasmid maintenance was assessed by determining plasmid yields and topologies after subculturing the transformed bacteria. Transfection efficiency was assessed by determining plasmid copy number, expression, and cytotoxicity after GET to mouse (B16-F10), canine (CMeC-1), and human (SK -Mel-28) melanoma cell lines.

Plasmid isolation yields of antibiotic resistance gene-free plasmids were on average lower than that of the commercial IL -12 plasmid. However, the yields remained constant after subculturing, confirming that these plasmids are stably maintained in transformed bacteria during cell division and are not lost over generations. After GET to melanoma cells, the number of plasmids detected in the transfected cells was relatively low (between 2 and 36 copies per cell), regardless of the plasmid used. Nevertheless, IL -12 expression from all four plasmids was detected in all three cell lines, confirming that even 2 copies per cell are sufficient for transgene expression.

We demonstrated that our mouse, canine, and human antibiotic resistance gene-free IL -12 plasmids are stably maintained in bacteria and support sufficient IL -12 expression after GET in vitro. The plasmids encoding mouse and human Il-12 orthologs have now proceeded to in vivo evaluation, while the plasmid encoding canine IL-12 is currently being used in a clinical study in client-owned dogs with various solid tumors.

P840

Biodistribution and re-targeting of ARRDC1-mediated microvesicles (ARMMs) for non-viral delivery of therapeutic payloads

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Intracellular delivery of large macromolecules such as RNA, protein, or genome editing payloads enables treatment of diseases across all therapeutic areas. The major hurdle to date has been the inability to identify a versatile vehicle that is conducive to safe and functional delivery of such payloads to multiple cell types and through different routes of administration. We report engineering of ARRDC1-mediated microvesicles as non-viral delivery vehicles that would potentially allow disease modification across multiple therapeutic areas and through targets defined as druggable or undruggable using more traditional agents. Intravenous administration of ARMMs in mice yielded uptake in liver sinusoidal endothelial cells, liver resident Kupffer cells, and marginal macrophages of the spleen. Intravitreal administration led to biodistribution to retinal ganglion cells in mice and rabbits, and subretinal administration to the retinal pigmented epithelium. Intranasal administration led to robust uptake in alveolar macrophages. We further surface engineered ARMMs to present an anti-CD8 antibody and demonstrate re-targeting of these vehicles to CD8+ T cells, suggesting that ARMMs can be adapted to deliver therapeutic payloads to a large number of target tissues and cell types.