enable the development of novel therapeutic interventions for rare and ultra-rare diseases to be more sustainable. To this aim, we tested the LVs encoding for each speci c enzyme (LV-GALNS, -enhGLB1, and -MAN2B1) in human HSPCs, showing that the progeny of transduced cells (myeloid cells and osteoclasts, serving as circulating and tissue-resident enzyme source) are capable of releasing supraphysiological amounts of the therapeutic enzyme, which is suf cient to cross-correct patients cells, including skeletal cells. Using the clinical-grade LV, we selected the platform transduction protocol showing high transduction ef ciency (VCN >2 and > 80% positive colonies) and no signs of toxicity in vitro and in vivo. A platform GLP study for parallel evaluation of enzyme overexpression toxicity is ongoing. In this study, we compare wild-type mice transplanted with murine hematopoietic stem cells transduced in vitro to overexpress each speci c enzyme to a single control group transplanted with un-transduced cells. Moreover, disease-speci c proof of concept studies started to evaluate the safety and ef cacy of HSPC-GT compared to standard HSPC transplantation. Our preliminary data support the platform development of HSPC-GT for LSDs and its clinical application.

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P0883

Pancreas-directed gene therapy for maturity-onset diabetes of the young type 3 (MODY3): Moving from mice to large animals

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Maturity-onset diabetes of the young (MODY) are a group of monogenic diabetes characterized by onset of hyperglycemia at an early adult age, normally before 25 years. MODY3, the most common type of MODY, is caused by mutations in the gene encoding for the transcription factor hepatocyte nuclear factor 1A (HNF1A). MODY3 patients are usually treated with sulfonylureas, but they tend to become unresponsive to these drugs and adverse effects associated to chronic administration of sulfonylureas have been reported. Presently, there are no current treatments addressing the root cause of MODY3. Here, using a unique new strategy based on the CRISPR/Cas9 technology, we generate a novel MODY3 mouse model that recapitulates the main alterations observed in human patients. Local administration of AAV vectors encoding Hnf1 to the pancreas of MODY3 mice via intraductal delivery resulted in Hnf1 expression in β-cells, which upregulated the expression of HNF1A main target genes, such as GLUT2 and L-PK. AAV-Hnf1 -mediated gene therapy counteracted hyperglycemia, increased insulinemia, improved glucose tolerance and enhanced insulin secretion by pancreatic β-cells, thus reverting the MODY3 phenotype. As a rst step towards translation, intraductal delivery of AAV vectors to Beagle dogs resulted in ef cient transduction of pancreas, including endocrine cells, in the absence of adverse events or toxicities. Overall, these results underscore the potential of the AAV-Hnf1 gene therapy to treat MODY3 and support its clinical translation.

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Profiling AAV integration in gene therapy and genome editing for Wilson disease

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Wilson Disease (WD) is an autosomal recessive genetic disorder caused by mutations in the *ATP7B* gene and characterized by toxic copper accumulation in liver and extrahepatic organs. Liver-directed gene therapy using adeno-associated viral (AAV) vectors offers potential treatment and is currently undergoing clinical trials. AAV genome typically remains episomal, but it could also integrate at low frequency in the host genome. While raising concerns in terms of genotoxicity, AAV integrants may also contribute to therapeutic effect. AAV integration preferentially occurred at DNA double-strand breaks (DSB) and we found that human *ATP7B*$^{-/-}$hepatocytes were more susceptible to DSB generation in response to copper challenge compared to wild-type cells. Consistently, increased DSBs were observed in ATP7B-de cient human and mouse livers relative to controls. Surprisingly, transcriptional upregulation of genes involved in homology-directed repair (HDR) was found in ATP7B-de cient human hepatocytes and mouse livers.

To investigate the effect of copper-mediated DNA damage on AAV integration, we analyzed the integration pro les of AAV-based classic gene therapy and genome editing in *Atp7b*$^{-/-}$ mice. Genome editing was achieved by nuclease-free targeted integration of a promoterless human mini-ATP7B cDNA into the albumin locus (Alb-ATP7B) through HDR. The gene replacement vector included a human mini-ATP7B cDNA under the control of a liver-speci c promoter (HLP-ATP7B). Control vectors bearing GFP (Alb-GFP and TBG-GFP) were also studied. AAV integration sites (IS) were retrieved from liver genomic DNA using Sonication Linker-Mediated (SLiM) PCR and the Recombinant Adeno-Associated Vector Integration (RAAVioli) pipeline.

Livers injected with the HLP-ATP7B vector displayed the highest IS number, a 100-fold increase compared to TBG-GFP controls, suggesting that these integration events provided a selective advantage of corrected hepatocytes. Conversely, similar IS numbers were observed in mice injected with genome editing vectors (Alb-ATP7B and Alb-GFP). Clonal diversity measured by the Shannon diversity index indicated higher polyclonality in HLP-ATP7B injected mice, whereas genome editing vectors showed a more oligoclonal pro le. Gene enrichment analysis revealed an over-representation of genes associated with liver damage and dysfunction. The albumin *(Alb)* gene was the most targeted in genome editing experiments and showed high targeting frequencies in HLP-ATP7B injected mice. However, IS distribution within the *Alb* gene varied depending on the vector used, with Alb-ATP7B integrations predominantly aligning with the direction of *Alb* gene transcription, thus potentially contributing to selective advantage. *Ppp1r12b*, *1700048O20Rik* and *Cps1* also emerged as frequently targeted genes by both gene therapy and genome editing vectors. TBG-GFP IS were often found in *Atp7a*, a paralogue of *Atp7b* with low hepatocyte expression, suggesting TBG-GFP integration may transactivate *Atp7a*, compensating for ATP7B function and conferring a selective advantage to hepatocytes. Consistently we found increased hepatic *Atp7a* expression in TBG-GFP-treated mice, compared to mice injected with HLP-ATP7B and to healthy controls.

In conclusion copper-mediated DNA damage may increase off-target and random integration events, but it could also enhance on-target integration of genome editing vectors through upregulation of HDR machinery. These findings underscore the importance of disease conditions in determining AAV integration profiles and highlight the need for further investigation into the safety of AAV-based therapies in the context of WD.

P0885

Development of *in vivo* genome editing for the treatment of progressive familial intrahepatic cholestasis type 2

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Progressive familial intrahepatic cholestasis type 2 (PFIC-2) is a monogenic inborn error of metabolism caused by the deficiency of the liver bile salt export pump (BSEP), the transporter of conjugated bile acids (BA) across the canalicular membrane of hepatocytes, encoded by *Abcb11*. PFIC-2 patients display cholestasis, jaundice, itching, and usually develop liver fibrosis and end-stage liver disease before adulthood. Current treatments consist of diet control, and pharmacological or surgical interventions aiming at decreasing BA blood concentration. However, most patients ultimately undergo liver transplantation as the only curative option. In diseases like PFIC-2, presenting with early onset and rapid disease progression, early therapeutic intervention would be fundamental to limit progressive damage to hepatocytes. Since BSEP expression is tightly regulated by BA levels through the farnesoid-X-receptor signalling, acting on *Abcb11* endogenous promoter, we are developing a liver-directed CRISPR-Cas9 based-genome editing approach aimed at integrating a corrective cDNA into the first intron of the *Abcb11* gene. This strategy may allow for correcting most of the disease-causing mutations while maintaining as much as possible the physiological gene regulation. Moreover, this approach may enable stable genetic correction even in pediatric patients at the first disease stages. We initially assessed LV-mediated liver gene addition in $Abcb11^{-/-}$mice, a PFIC-2 model, to correlate therapeutic effects with the extent of liver modification. We administered 2-week-old $Abcb11^{-/-}$mice with LV encoding the murine BSEP transgene under the control of a hepatocyte-specific cassette based on the enhanced transthyretin promoter. We observed that transgene mRNA levels of about 30% of the endogenous murine BSEP resulted in a significant decrease, up to normalization, of serum BA, bilirubin, and alkaline phosphatase biomarkers until 6 months of age in both males and females, indicating prevention of cholestatic damage. Towards genome editing, we selected the most efficient *Abcb11*-targeting guide RNA (gRNA) in hepatocyte cell lines. Moreover, we compared different mCherry-expressing donor DNAs designed to exploit homology-directed repair (HDR) or homology-independent targeted integration (HITI) pathways for site-specific integration in the first intron of *Abcb11* gene. We achieved the highest integration rate when using the HDR-based donor DNA (up to 12%). *In vivo*, we tested the different mCherry-donor DNA configurations delivered via adeno-associated viral vectors (AAVs) to 2-day or 2-week-old Cas9 transgenic mice. In the liver of mice of both ages, we observed a slightly higher integration rate (up to 12%), mCherry expression, and mCherry-positive liver area (up to 14%) exploiting homology-dependent rather than -independent pathway. The treatment of 2-week-old wild-type mice with AAV-donor DNA, followed by administration of lipid nanoparticles carrying Cas9 mRNA and gRNA, confirmed higher efficiency of the HDR-based donor construct compared to HITI. We are currently testing the selected editing reagents in $Abcb11^{-/-}$mice to evaluate both the liver site-specific integration rate and therapeutic efficacy in the mouse model of PFIC-2, treated early in life. Overall, these studies will inform about the feasibility and efficacy of *in vivo* liver-directed genome editing for the treatment of PFIC-2.

P0886

Hematopoietic Stem Cell Gene Therapy for Hurler Syndrome (OTL-203): interim skeletal, neurological and systemic outcomes

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Preliminary results of an ongoing phase I/II study of OTL-203, an autologous hematopoietic stem gene therapy (HSC-GT, NCT03488394), in 8 Mucopolysaccharidosis type I Hurler syndrome (MPSIH) patients suggest superior metabolic correction and initial clinical response compared to allogeneic haematopoietic stem cell transplantation (HSCT) published in literature (Gentner *et al. N Engl J Med* 2021; *Consiglieri et al. Sci Transl Med 2024*). Here we report interim skeletal, neurological and systemic outcomes up to 4-year after HSC-GT.

IDUA activity was measured in blood and cerebral spinal fluid (CSF), dermatan (DS) and heparan sulphate (HS) in urine and CSF at baseline and different timepoints up to 4-year after OTL-203 treatment. Skeletal, neurological, corneal clouding (CC), carpal tunnel syndrome (CTS) and cardiac outcomes were assessed at baseline and different timepoints up to 4-year after treatment. For specific outcomes OTL-203 patients were compared with an external cohort of MPSIH patients who underwent HSCT.

IDUA activity in blood reached supraphysiologic levels in all patients from 30 days after OTL-203 treatment resulting in urinary substrate clearance, maintained at last follow-up. All patients exhibited longitudinal growth within expected reference ranges according to age and gender with a median height gain greater than HSCT patients and experienced an earlier normalization of joint mobility compared with HSCT patients. Both hip X-ray and MRI parameters improved after OTL-203 treatment, underlining an amelioration of hip dysplasia up to last follow-up. A specific spine MRI score showed stabilization of the typical spine MRI features at last follow-up. From 3 months after OTL-203 treatment, IDUA activity became detectable in CSF and persisted overtime accompanied by local reduction of DS/HS (undetectable DS in 7/8 patients at 4-year follow-up). After OTL-203 treatment, 7/8 patients continued to gain motor and cognitive skills in line with typically developing children and experienced an improvement of typical brain MRI abnormalities. At baseline, all subjects had evidence of mild to moderate CC. Of the OTL-203