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REVIEW

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Liver-directed gene therapy for inherited metabolic diseases

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Abstract

Gene therapy clinical trials are rapidly expanding for inherited metabolic liver diseases whilst two gene therapy products have now been approved for liver based monogenic disorders. Liver-directed gene therapy has recently become an option for treatment of haemophilias and is likely to become one of the favoured therapeutic strategies for inherited metabolic liver diseases in the near future. In this review, we present the different gene therapy vectors and strategies for liver-targeting, including gene editing. We highlight the current development of viral and nonviral gene therapy for a number of inherited metabolic liver diseases including urea cycle defects, organic acidaemias, Crigler–Najjar disease, Wilson disease, glycogen storage disease Type Ia, phenylketonuria and maple syrup urine disease. We describe the main limitations and open questions for further gene therapy development: immunogenicity, inflammatory response, genotoxicity, gene therapy administration in a fibrotic liver. The follow-up of a constantly growing number of gene therapy treated patients allows better understanding of its benefits and limitations and provides strategies to design safer and more efficacious treatments. Undoubtedly, liver-targeting gene therapy offers a promising avenue for innovative therapies with an unprecedented potential to address the unmet needs of patients suffering from inherited metabolic diseases.

KEYWORDS

genome editing/insertion, inherited metabolic disease, liver gene therapy

1 | INTRODUCTION

Several inherited metabolic diseases are an indication for orthotopic liver transplantation (OLT) that replaces thousands of liver enzymes from the donor to correct a single enzyme defect. Inherited metabolic diseases corrected by OLT are targets also for liver-directed gene therapy that by precise and non-invasive approach corrects the single disease-causing gene defect.

In the last two decades, adeno-associated virus (AAV) vectors have been employed for liver targeting with preclinical and clinical data supporting their efficacy without significant toxicity. Other approaches using viral and nonviral vectors have also been developed. In this review, we will discuss the approaches that have been investigated for liver-directed gene therapy, the vectors and the disease targets illustrating both the success and the limitations in the context of inherited metabolic diseases.

2 | GENE THERAPY VECTORS

Liver gene therapy has been performed both ex vivo and in vivo. Ex vivo gene therapy is performed in combination with hepatocyte transplantation when diseased liver cells from the patient genetically corrected outside the body are reinjected into the same patient (autologous cell transplantation). In contrast, in vivo liver-directed gene therapy is based largely on intravenous injections, albeit hepatic artery injections have been performed in some older clinical trials. Ex vivo gene transfer is effective in disorders in which expression of the therapeutic gene provides a potential selective growth advantage over uncorrected cells.[1] However, outcomes of ex vivo liver gene therapy have been disappointing because of limited and short-lasting engraftment of genetically modified hepatocytes, and lack of proliferative advantage of corrected hepatocytes for most inherited metabolic liver diseases. Moreover, ex vivo approaches for liver gene therapy are also complicated by the need for repeated removal of the hepatic tissue to obtain the hepatocytes and reinfusion of genetically modified cells via the portal vein, and the limited viability of cultured primary hepatocytes.[2] These limitations were the reasons for the unsuccessful outcome of the ex vivo hepatocyte gene therapy in the clinical trial performed almost 30 years ago for familial hypercholesterolemia due to homozygous *LDLR* mutations.[2] Based on these considerations, gene therapy for inherited metabolic liver diseases relies on in vivo approaches. AAV vectors have shown the greatest clinical success for in vivo liver gene therapy so far. The mechanism of cell transduction by AAV vectors is not fully understood but is likely to occur through receptor-mediated endocytosis after which AAVs are able to escape the endosomes. AAVs are inefficient at integration into the host cell genome and therefore, the transgene is expressed via 'episomes' i.e., extra-chromosomal DNA molecules that are not efficiently transmitted to daughter cells after division.

AAV vectors can accommodate sequences up to 4.5–5 kb in size, whereas the inclusion of sequences greater than 5 kb reduces significantly the in vivo potency.[3] This limited cargo capacity of AAV vectors has been a major hurdle for haemophilia A, Duchenne muscular dystrophy and Stargardt disease, but with few exceptions (e.g., carbamoyl phosphate synthetase 1 [CPS1]) most inherited metabolic diseases are due to defects in genes that can be accommodated by AAV. Multi-year expression of the therapeutic gene has been observed after a single AAV vector administration into post-mitotic or slowly replicating adult tissues.[4]

Subjects recruited to liver-directed gene therapy clinical trials have been for the most part adults with fully grown liver. In this case the effective AAV-mediated hepatocyte transduction is not lost by mitotically active and growing livers. In contrast, vector administrations in animal models undergoing liver growth were associated with loss of transgene expression, indicating that full AAV vector genomes do not integrate in a significant proportion of the host cells and transgene expression is lost in actively replicating cells.[5] Although a single administration of AAV vector may be sufficient to achieve a lifelong correction in diseases with low therapeutic threshold such as haemophilia, vector dilution is a limitation in several inherited metabolic diseases requiring higher percentages of hepatocyte transduction or interventions in early childhood.[6]

Although morphologically similar, hepatocytes differ in their metabolic functions along the porto-central axis. These differences include differential expression of enzymes involved in metabolism of carbohydrates, amino acids, ammonia, lipids, and bile formation.[7] Because liver zonation plays an important role in liver metabolic functions, this transduction bias is likely to be relevant for gene therapy of inherited metabolic diseases. Whilst in mouse and dog livers, it occurs predominantly in pericentral regions, in non-human primate livers AAV transduction is mainly in periportal regions[8] and in humans is unknown (Figure 1).

Although largely used for ex vivo gene therapy, phagocytosis-shielded lentiviral vectors (LVs) administered in vivo by intravenous administrations were found to be efficient in liver gene transfer of nonhuman primate (NHP) livers without signs of toxicity or clonal expansion of transduced cells.[9] This approach overcomes the limitations due to increased immunogenicity and low levels of transgene expression from the non-shielded LV vectors.[10] Moreover, the efficient genomic integration of LV vectors gives them an advantage over AAV vectors when targeting growing livers. Furthermore, the lower prevalence of HIV antibodies in the population makes LV attractive vectors for a broader patient population. However, LV vectors have never been administered in vivo in humans and the risks of systemic injections are completely unknown. Moreover, risks related to insertional mutagenesis by this route of administration are also unknown.

A growing number of preclinical and clinical studies have shown that mRNA encapsulated in lipid nanoparticles (LNPs) and injected systemically can efficiently target the liver.[11] In contrast to viral vector vectors, LNPs are weak at delivering DNA to the nuclei of hepatocytes but compared to DNA gene therapy, mRNAs do not require transit to the nucleus, thereby mitigating genotoxicity risks. The LNPs protect the mRNA from nuclease-mediated degradation and shields it from the immune system. LNPs are internalized into hepatocytes

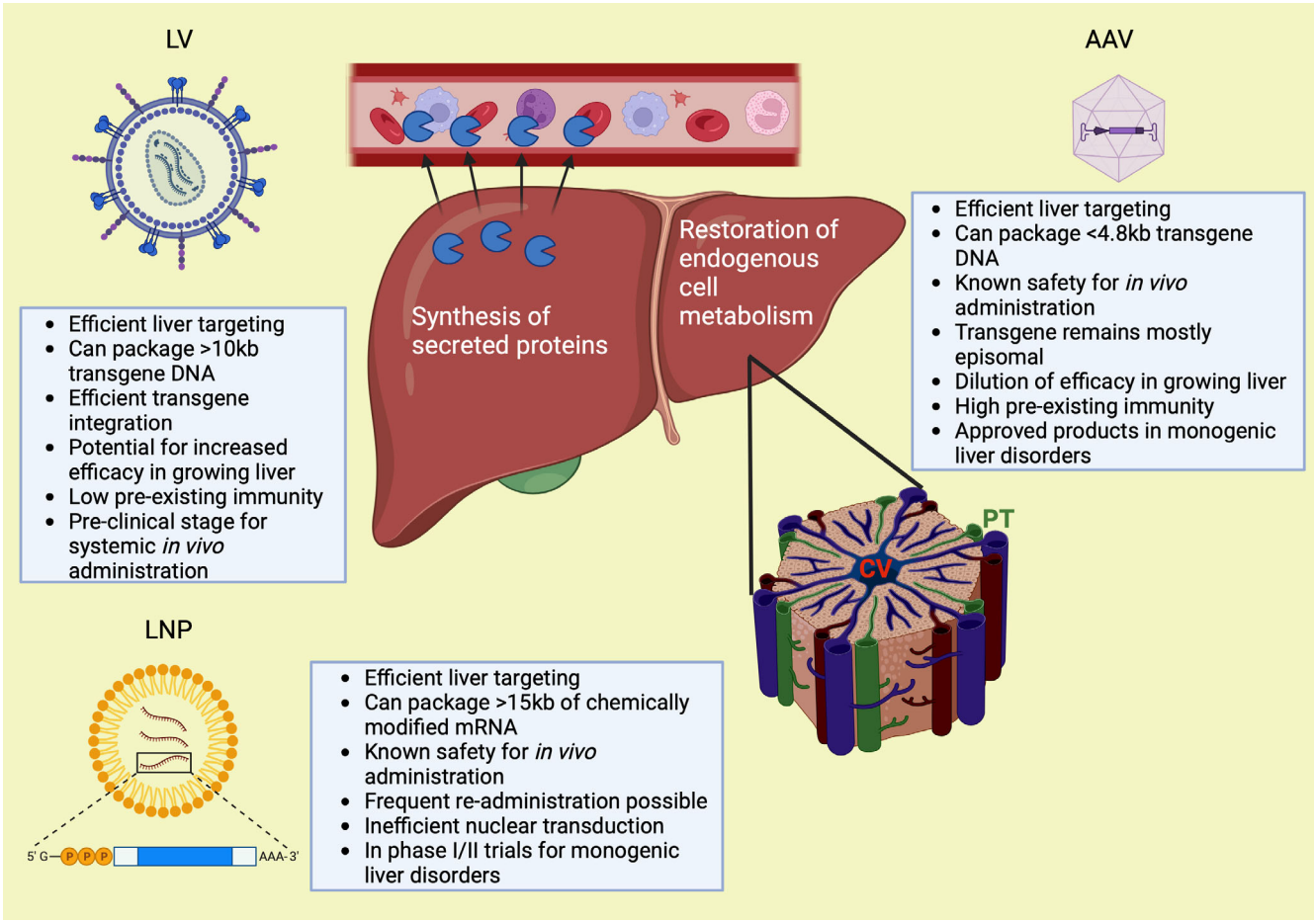

FIGURE 1 Main vectors and goals of liver-directed gene therapy. AAV, adeno-associated virus; CV, central vein; LNP, lipid nanoparticle; LV, lentiviral vector; PT, portal tract. Created with biorender.com.

and once in the endosome, ionizable lipids induce endosome escape and release of the mRNA cargo into the cytoplasm. However, mRNA provides transient, half-life-dependent protein expression and therefore requires repeated lifelong administrations[11] (Figure 1).

3 | GENE THERAPY STRATEGIES

Gene replacement or *gene augmentation* is based on the addition of a normal copy of the mutated gene. Gene editing entails *gene correction*, by which a pathogenic variant can be edited and therefore 'corrected', or *gene insertion*, which enables insertion of the whole therapeutic gene or expression cassette at a desired locus in the host genome. Gene correction is a mutation-specific technology, whereas gene insertion is mutation-independent. Gene correction can be achieved by various nuclease-mediated approaches, which are based on genomic site-specific recognition. The most common technology is based on the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR-Cas9) coupled to guide RNA, which creates a double-strand break (DSB) at a desired locus.[12] The DSB is corrected using a DNA template, which contains the wild-type DNA sequence, through the process of homology-directed repair (HDR) that is restricted to dividing cells. In the absence of DNA template, the DNA repair mechanism is based on non-homologous end joining (NHEJ), that generates insertions and deletions (indels) for gene inactivation.[13] Base editing employs cytosine base editor (CBE) or adenine base editor (ABE)[14] that are deaminases linked to a Cas9 nickase and CRISPR guide RNA to modify a base pair at a specific locus. CBE will convert cytidine to uracil and ultimately, C–G to T–A. ABE-mediated editing converts A–T to G–C base pair. Prime editing fuses an engineered reverse transcriptase, Cas9 nickase and a prime editing guide RNA. This technology enables correction of a small deletion or insertion or missense mutation without creating DSB that are error-prone.[15] Programmable addition via site-specific targeting elements (PASTE) fuses reverse transcriptase, Cas9 nickase and serine integrase. This

technology can insert large payloads in dividing and non-dividing cells.[16] CRISPR-Cas9 editing, base editing and prime editing have all shown proof-of-concept evidence of efficacy in preclinical models of inherited metabolic liver diseases. For example, CRISPR-Cas9 editing has been used to correct missense pathogenic variant in ornithine transcarbamylase (OTC)-deficient (*Spf*^{ash}) mice using HDR, restoring survival and ureagenesis.[17] Alternatively, CRISPR-Cas9 was used to inactivate non-essential hepatic genes by NHEJ, acting as substrate reduction therapy in disorders due to accumulation of toxic metabolites, such as primary hyperoxaluria Type I.[18] Moreover, this strategy showed clinical benefit in patients affected by transthyretin amyloidosis due to accumulation of misfolded transthyretin protein encoded by mutated *TTR* gene. *TTR*-targeting guide RNA and CRISPR-Cas9 mRNA delivered by LNP and administered intravenously, led to significant reduction of plasma TTR concentrations in adult patients, thereby preventing the synthesis of toxic transthyretin.[19] Base editing and prime editing have shown proof of concept in a preclinical model of phenylketonuria (PKU).[20,21]

Gene integration can be achieved by homologous recombination without nuclease but at very low rate and correction of the disease phenotype can only be obtained if selective advantage results in liver repopulation by hepatocytes with the integrated therapeutic gene. The homology arms flank the transgene, thereby enabling the integration at the selected locus. Another approach is to use homologous recombination with a nuclease to create a DSB, which will significantly increase the rate of integration events. These nucleases can be site-specific, using Cas9 guide RNA, or non-specific, cutting at a simpler motif like piggyback transposase. All gene integration strategies have shown efficacy in preclinical models of inherited metabolic liver diseases.[22–24]

4 | DISEASE TARGETS FOR LIVER-DIRECTED GENE THERAPY

Several inherited metabolic diseases have been considered as targets of liver-directed gene therapy. The most attractive targets are disorders that are severe to justify the risks of new therapies and have high prevalence. Moreover, attractive candidates are disorders that can be fully corrected by liver gene transfer and require small percentage of hepatocyte correction to achieve clinical benefit. The amount of correction ultimately depends on the magnitude of the metabolic flux through the biochemical pathway on a cellular level and on the organ level.

4.1 | Urea cycle disorders

Because liver transplantation remains the only curative approach, several urea cycle disorders have been proposed as candidates for liver-directed gene therapy. For CPS1 deficiency, the size of CPS1 gene and the limited AAV packaging capacity required a split-approach with two AAV vectors.[25] Nevertheless, in preclinical studies for CPS1 deficiency as well as other urea cycle disorders, AAV have shown phenotype correction with increased survival, improved growth, normalization of ammonia and plasma amino acid concentrations (reviewed by Duff et al.[26]). The preclinical studies in OTC deficiency have led to a first-in-human Phase I/II AAV8 gene therapy clinical trial for adults with late-onset OTC deficiency sponsored by Ultragenyx (NCT02991144), with seven patients considered as responders out of 11 treated. A Phase III trial is now enrolling (NCT05345171). Moreover, another clinical trial targeting paediatric OTC deficiency using the hepatotropic AAV-LK03 vector and sponsored by University College London (NCT05092685) is at a recruiting stage after having shown safety in NHP[27] and reduced seroprevalence for AAV-LK03 capsid.[28] By comparing plasma FVIII concentrations in haemophilia A clinical trials using comparable vector doses,[29,30] the AAV-LK03 appears to transduce human hepatocytes a log-better than AAV8, consistent with the observations made by other studies.[31–33]

The limitations of AAV gene therapy have emerged in the treatment of urea cycle disorders in neonatal animal models.[34] Lack of efficacy of AAV re-administrations due to anti-AAV immune response[35] fostered the need for novel strategies, either based on genome editing or non-viral vector-mediated delivery. Gene correction was successfully performed in OTC-deficient mice by CRISPR-Cas9 editing of a single missense mutation, correcting the hyperammonaemia.[36] However, due to a lack of common mutations in urea cycle disorders, mutation-specific editing approaches would only benefit a small number of patients. To overcome this problem, whole transgene integration strategy has been developed. A nuclease-mediated widespread integration in the host genome used piggyback transposase with phenotypic correction in citrullinemia and OTC-deficient mice.[23] Alternatively, transgene integration can be performed at a specific locus of the host genome by nuclease-mediated integration with homologous recombination sequences mapping the integration locus and flanking both extremities of the transgene. This has been successfully performed in OTC-deficient mice.[17,24]

Non-viral gene therapy using repeated systemic injections of LNPs encapsulating mRNA has shown efficient

liver targeting and phenotypic correction of mouse models of OTC deficiency,[37,38] argininosuccinate lyase deficiency[39,40] and arginase deficiency.[41,42]

4.2 | Crigler–Najjar syndrome

Crigler–Najjar syndrome due to deficiency of the enzyme uridine diphosphoglucuronate glucuronosyltransferase 1A1 (UGT1A1) has long been considered an excellent target for liver-directed gene therapy. The lack of UGT1A1 results in severe unconjugated hyperbilirubinemia that can cause irreversible neurologic injury and death. Prolonged, daily phototherapy partially controls the jaundice, but the only definitive cure is liver transplantation. Recent results of the dose-escalation portion of a Phase I/II study of AAV8 based gene therapy have shown sustained serum bilirubin concentrations below the toxic threshold allowing discontinuation of phototherapy in the high-dose cohort.[43] The same concerns as in OTC deficiency have emerged also for liver-directed gene therapy of Crigler–Najjar syndrome in neonatal/paediatric patients because the progressive loss of episomal AAV viral genomes over time, due to hepatocyte proliferation, results in the reduction of both transgene expression and efficacy of the treatment. Like OTC deficiency, to overcome these obstacles and to achieve long-lasting efficacy, the insertion of UGT1A1 after the albumin promoter using CRISPR/SaCas9 resulted in long-term correction.[44] This approach allows (i) higher gene-targeting rate; (ii) higher levels of transgene expression; (iii) permanent modification of the genome with life-long therapeutic efficacy; (iv) a mutation-independent approach because the AAV vectors can be used for all disease-causing *UGT1A1* mutations. However, there are also limitations such as the requirement of two independent AAV vectors, one to deliver the Cas9 and guide RNA and the other containing a promoterless *UGT1A1* flanked by albumin homology regions. Despite the great potential, CRISPR/Cas9 still holds several safety and efficacy concerns limiting clinical development. Off-target effects has been one of the main issues that led to the development of more precise Cas9 and improved gRNA design.[45] Nevertheless, unexpected off-target effects may still occur and are difficult to predict using the human genome reference sequence that does not take into account the human genome diversity.[46] Genome editing by CRISPR/Cas9 is inhibited by tumour suppressor p53 and is increased when p53 is mutated.[47–49] These findings raised the concern that CRISPR/Cas9 may lead to expansion of p53-inactivating mutations in corrected cells and ultimately in cancer.

An additional barrier is the pre-exiting immunity against Cas9 because recent studies detected circulating antibodies against SpCas9 and SaCas9 and memory T cells against SpCas9 in the majority of human adult individuals.[50,51] Although this is not surprising given that bacterial strains expressing these Cas9 frequently infect humans, pre-exiting immunity may limit in vivo applications of AAV-delivered Cas9 by triggering a cytotoxic T-cell response against Cas9-expressing hepatocytes and loss of edited cells.[52] Potential solutions for evading the immune response toward CRISPR-Cas9 include the use of CRISPR-Cas systems to which humans have not previously exposed[53] or transient delivery of Cas9 mRNA or protein by targeting chemically modified LNPs.[54,55]

4.3 | Organic acidemia

Organic acidemias, especially methylmalonic (MMA) and propionic acidemias (PA), have been the targets of several preclinical gene replacement therapy studies, and they have been recently reviewed by Chandler and Venditti[56]. Whole transgene integration has been successfully performed using a promoterless AAV vector without nuclease using homologous recombination to target the albumin locus of the host genome. This phenotypic correction was observed in both neonatal and juvenile MMA mouse models.[22,57] Although the percentage of corrected hepatocytes was low, a selective advantage showed partial progressive repopulation of the native MMA liver by edited hepatocytes, from 2% to 15% over 15 months. The phenotype was not fully corrected with persistence of severely impaired growth. This preclinical work led to a Phase I/II clinical trial sponsored by Alexion Pharmaceuticals with an AAV-LK03 capsid (NCT04581785). In this trial two patients developed thrombotic microangiopathy (TMA) not predicted by preclinical murine studies which resolved with supportive treatment. The clinical trial was placed on temporary hold by the FDA and then terminated.

LNP-mRNA therapy has been successfully performed in both neonatal lethal[58] and juvenile[59] MMA mouse models. Pharmacokinetics showed week-long persistence of MMUT protein expression following systemic administration and weekly repeated administrations for 5 weeks showed phenotypic correction with no safety issues. These studies led to a Phase I/II clinical trial sponsored by Moderna Therapeutics currently recruiting pediatric MMA patients (NCT04899310). The same therapeutic platform showed proof of concept in fibroblasts from PA patients[60] and hypomorphic PA mice.[61] Interestingly, this approach used a dual mRNA approach with both *PCCA*

and *PCCB* mRNAs encapsulated in biodegradable LNPs to treat PA phenotype from the two gene defects resulting in PA. This work led to translation with an ongoing Phase I/II clinical trial sponsored by Moderna Therapeutics currently recruiting pediatric PA patients (NCT04159103).

4.4 | Wilson disease

Wilson disease due to defects in the *ATP7B* gene is treated with copper chelators and zinc salts, which have side effects and do not normalize copper metabolism. AAV gene therapy using AAV8 and encoding the full length human *ATP7B* gene was tested in Wilson disease adult mice and enabled restoration of physiological biliary copper excretion in response to copper overload and absence of liver histological alterations.[62,63] As the *ATP7B* gene is 5.2 kb and reaches the maximal AAV packaging capacity, a *miniATP7B* gene, in which four out of six metal-binding domains were deleted from the wild-type *ATP7B* coding sequence, was packaged in an engineered hepatotropic AAV-Anc80 capsid and successfully tested in Wilson disease adult mice with similar efficacy at improving survival, restoring copper homeostasis and preventing liver damage.[64,65] A split-approach with two AAV vectors encoding each half of the wild-type transgene with a homologous recombination strategy to obtain the full length ATP7B protein was also successfully tested in Wilson disease mice.[66]

These preclinical studies paved the way for liver-directed AAV clinical trials targeting Wilson disease. Two studies are currently recruiting adult Wilson disease patients with stable liver disease: i) the GATEWAY study, sponsored by Vivet Therapeutics, is assessing the safety and efficacy of VTX-801, an AAV-Anc80 capsid encoding the *miniATP7B* gene, in a phase I/II clinical trial (NCT04537377); ii) the Cyprus^{2+} clinical trial, sponsored by Ultragenyx, is assessing safety and efficacy of UX701, an AAV9 vector encoding a *miniATP7B* gene (NCT04884815). Interestingly, recent preclinical data showed that AAV-mediated liver-directed, nuclease-free genome editing targeting integration of a promoterless *miniATP7B* into the Albumin locus resulted in extensive liver repopulation as a consequence of a proliferative advantage over non-edited cells.[67]

4.5 | Glycogen storage diseases Type 1a

With growing evidence of safety and efficacy of AAV-mediated clinical gene therapy, the number of disease targets for gene therapy is rapidly increasing to include diseases with available but still cumbersome therapies, such as glycogen storage disease type Ia (GSDIa), maple syrup urine disease (MSUD) and PKU. GSDIa is typically managed with nutritional therapy to maintain normal blood glucose concentrations, prevent hypoglycaemia and provide optimal nutrition for growth and development. Liver-directed, AAV-mediated delivery of the gene encoding glucose-6-phosphatase (G6PC) that is defective in GSDIa, prevented hypoglycemia in murine and canine models of GSDIa, even at low levels of expression (3%–5% of wild-type activity). However, vector genome loss due to liver regeneration, the need of simultaneous targeting of liver and kidney and unknown long-term hepatic complications (e.g., liver tumours) have been raised as concerns for AAV-mediated gene therapy. Nevertheless, preliminary data from an ongoing Phase 1/2 clinical trial (NCT03517085) suggested some degree of efficacy with increased fasting tolerance. Gene therapy for these disorders have been recently reviewed in this journal by Koeberl et al.[68] Non-viral mRNA therapy has also shown proof of concept in GSDIa,[69] paving the way for a Phase 1/2 clinical trial by Moderna (NCT05095727), currently recruiting adult and pediatric patients affected by GSDIa.

4.6 | Maple syrup urine disease

MSUD is due to deficiency of the branched-chain 2-keto acid dehydrogenase, a multimeric enzyme complex with four components: E1α and E1β, dihydrolipoyl transacylase (E2) and dihydrolipoamide dehydrogenase (E3) subunits. In the classical severe form of MSUD, with less than 3% residual enzyme activity, leucine accumulation causes coma and cerebral edema shortly after birth with early death in the absence of aggressive and timely management. A single AAV vector infusion in neonatal MSUD mice, with either E1α or E1β defects, resulted in long-term survival of the animals and rescue of the disease phenotype.[70,71] Liver-restricted gene transfer by a liver-specific promoter provided partial correction of the MSUD phenotype, suggesting that extrahepatic expression of the enzyme is needed to achieve full therapeutic efficacy. Long-term disease rescue was indeed obtained with an ubiquitous promoter, consistent with the branched-chain amino acid oxidation that takes place in several tissues, especially the muscle.

4.7 | Phenylketonuria

Various gene therapy approaches for PKU have been reviewed by Martinez et al.[72] A sustained phenotypic correction was observed in preclinical models.

To tackle the rapid episomal loss of AAV-mediated transgene expression in a growing liver, integrative strategies have been developed with in vivo lentiviral gene therapy providing a selective advantage of transduced hepatocytes by inhibiting the Cypor enzymatic system involved in the protection from paracetamol-related toxicity. Cypor inhibition in a context of repeated paracetamol exposure enabled a positive selection of transduced hepatocytes to repopulate the native liver and phenotype correction in PKU mice.[73]

A gene editing approach using a dual AAV8 system has shown efficacy in correcting the missense mutation carried by the *Pah*$^{enu2/enu2}$ mouse using Cas9 and HDR, but conveyed a low frequency of edited hepatocytes (from 1% to 13%), enabling only a partial reduction of systemic phenylalanine concentrations.[74] Base editing with CBE delivered by AAV8 vector was successful in correcting the missense mutation of PKU mice in >20% hepatocytes with full correction of the phenotype and normalization of phenylalanine levels.[21] A similar result was achieved with a base editor delivered as LNP-encapsulated mRNA.[75] Prime editing enabled to treat neonatal PKU mice with 11% of corrected hepatocytes, however, using the highly immunogenic adenoviral vector at high doses, which limits its translation with this vector.[20]

Non-viral strategy using LNP-mRNA requiring repeated systemic administration has shown proof of concept in PKU mice.[76,77]

5 | OPEN ISSUES WITH AAV LIVER GENE THERAPY

5.1 | Immunologic responses

Seroprevalence of neutralizing antibodies directed against natural AAV in humans varies geographically and with ageing.[78] Systemic delivery of AAV vectors in the presence of such neutralizing antibodies fail to achieve clinically relevant liver transduction.[79,80] Therefore, subjects positive for neutralizing antibodies are excluded in most trials. Moreover, long-term persistence of high-titre, multi-serotype cross-reactive AAV neutralizing antibodies have been detected post-AAV vector systemic administration,[81] which prevents vector re-administration, that might be needed if transgene expression is lost. Multiple strategies have been investigated to overcome the neutralizing anti-AAV antibodies ranging from capsid switching, plasmapheresis, and pharmacological modulation of B- and/or T-cell activation.[82] Interestingly, rapamycin encapsulated particles co-administered with AAV vectors, prevented the induction of anti-capsid humoral and cell-mediated responses, thus allowing effective re-administration in mice and nonhuman primates.[83] Treatment with imlifidase, an endopeptidase used in transplanted patients[84] that degrades circulating IgG, also resulted in enhanced liver transduction in mice and nonhuman primates, when administered before AAV vector infusion, enabling vector re-administration.[85] Although promising, these strategies will need to be trialed in patients to demonstrate their efficacy.

A different immunologic issue of AAV gene therapy is a T-cell response to AAV capsid occurring after the intravenous injections of AAV vectors.[4,86,87] About 4 weeks after vector infusion, an asymptomatic increase in liver transaminases (alanine aminotransferase [ALT] and aspartate aminotransferase [AST]) peaking at about 6 weeks and returning to normal levels by 8 weeks without any medical intervention was observed concomitantly with loss of transgene expression.[4,86,87] Short-term treatment with glucocorticosteroids appears to be effective in blunting this T-cell immune response, controlling liver transaminases, and allowing long-term expression of the therapeutic gene.[87] However, monitoring of ALT and AST levels after gene therapy in patients with liver diseases resulting in increased serum transaminase activities, such as GSD1a and Wilson disease, remains problematic.

5.2 | Inflammatory response.

In SMA patients AAV vector doses of $5 \times 10e^{13}$ vector genomes (vg)/kg or higher have been associated with a complication of TMA[88,89] and one patient died as a result of TMA.[90] These vector doses are significantly higher than doses required for liver gene transfer needed to achieve therapeutic benefit in inherited metabolic liver disorders. TMA after AAV injections was associated with complement activation and different treatments were used. In some cases, supportive intravenous fluid administration was sufficient but some patients required plasmapheresis, steroids, haemodialysis, platelet transfusion, and eculizumab, a complement inhibitor. In addition, progressive and lethal cholestatic liver disease was also found in four patients with X-linked myotubular myopathy, a severe neuromuscular disease associated with hepatobiliary disease, who died after receiving an AAV8 at a dose of $1.3 \times 10\ e^{14}$ and $3.5 \times 10\ e^{14}$ vg/kg.[91–93] Whether the underlying liver disease contributed to this liver toxicity is unclear. With high AAV vector doses injected systemically, toxicity becomes a clinical concern, as shown by clinical trials for spinal muscular atrophy and Duchenne muscular dystrophy.[88,94] AAV hepatotoxicity is a common adverse event mediated by either immediate innate immune response or T-cell-mediated adaptive

immune response occurring some weeks after AAV administration, which can be mitigated by immunomodulation.[95] Liver complications are more likely to happen in diseased liver.[93] However, the mechanisms underlying these toxicities at high AAV vector doses remain largely unknown.

5.3 | Genotoxicity

The risk of genotoxicity driven by gene therapy relies on the insertion of foreign DNA sequences in the host genome, which can act as promoter or enhancer and interfere with the expression of oncogenes. This risk is carefully assessed in all preclinical studies. Patients, who have received gene therapy vectors, which have a potential for DNA integration, even at low frequency, require long-term follow-up to monitor this theoretical risk of insertional mutagenesis.[96] Oncogenic events in patients treated with gene therapy have been recognized for many years. Secondary leukemias and myelodysplastic syndrome have been reported after integrating gene therapy (gammaretroviral or LVs) occurred in clinical trials targeting haematopoietic stem cells to treat severe combined immunodeficiency syndrome[97,98] or sickle cell disease.[99] AAV vectors have an integration rate of 1%–3%[100] and have been associated with oncogenic events with no confirmed evidence of insertional mutagenesis in humans.[101,102] Hepatocellular carcinoma (HCC) has been observed in patients receiving AAV gene therapy but without demonstrated causative effect. Investigations of a haemophilia B patient recruited in an AAV clinical trial, who developed HCC, concluded that the AAV vector was unlikely to be the cause for HCC.[102]

The assessment of the oncogenic risk associated with AAV gene therapy is complex because this risk is influenced by the dose of vector, the promoter activity and the patient's age at injection.[103–105] Most of the oncogenic events observed in animal testing were due to AAV insertions in a mouse-specific *Rian* locus, which encodes a long-non-coding RNA that plays a role in epithelial to mesenchymal transition, regulation of Notch signalling and hepatic stellate cell activation. *Rian* is an ortholog of the human long-non-coding RNA MEG8, correlated with poor prognosis in human HCC, supporting the causative role of dysregulated *Rian* in tumourigenesis. In long-term studies of haemophilia A[106] and B[107] dogs, no genotoxicity was observed. Two haemophilia A dogs did show clonal expansion of transduced hepatocytes 4 years after treatment with a large number of insertions (>40%) identified close to oncogenes.[106] In NHP preclinical safety studies, no genotoxic effect of AAV has been observed, although these animals were observed only for a short period of time. Integration site analysis in NHP have reported widespread integration with no clonal expansion.[108–110] More recently, broadly distributed genomic integrations of vector sequences, including complex concatemeric structures, were detected in about 1% of liver cells of NHP.[111]

Sequencing of human HCC have revealed the clonal expansion of wild type AAV2 sequences within HCC-recognised oncogenes with a low prevalence.[112] An additional study identified wild-type AAV insertions in 8% of liver tumours and confirmed recurrent clonal AAV insertions in HCC-related oncogenes in non-cirrhotic livers.[113] Using an unbiased, next-generation sequencing-based approach, genomic integrations of AAV or wild-type AAV showed similar, broad distribution patterns, with a higher frequency in regions vulnerable to DNA damage or with highly transcribed genes.[114]

Overall, these findings suggest a theoretical risk of genotoxicity of AAV vectors. This risk is likely to be low, especially given the cumulative safety experience with the exponential number of liver-targeting AAV trials, combined with the low rate of HCC-associated AAV integrations despite the high seroprevalence of wild-type AAV in the human population (e.g. >50% for AAV2).[115] Expert recommendations from regulators endorse 'the current lack of observed AAV-associated HCC in large animal models and humans', suggesting 'a low risk compared to neonatal mice'. In conclusion, the number of AAV-treated human subjects remains small, and careful follow-up and surveillance of subjects enrolled in AAV gene therapy clinical trials is recommended.

Genotoxicity is a concern particularly for inherited metabolic diseases with increased cancer risk (e.g., GSDI, GSDIII, GSDIV, tyrosinemia Type 1, mitochondrial DNA depletion, and citrin deficiency). In the context of these disorders, the additional risk of insertional mutagenesis and inflammation induced by AAV vectors is a concern. However, correction of the metabolic defect in at least a subset of the liver cells is also expected to improve the metabolic defect, thus reducing the burden of toxic metabolites associated with increased cancer risk.

5.4 | Gene transfer to diseased livers

Several inherited metabolic liver diseases that are candidates for gene therapy can present significant liver abnormalities at the time of gene therapy administration. This can entail cholestasis, hepatitis, fibrosis, cirrhosis, adenomas or liver tumours. Little is known about gene transfer to diseased liver. Chronic liver inflammation was suggested to reduce the expression of AAV-mediated transgene expression[116] and could be an additional risk factor

for HCC following AAV gene therapy.[117] Liver remodelling, especially in cirrhosis with regenerative nodules, is likely to impact the biodistribution of systemically administered gene therapy vectors. It is important to separately investigate the risk of genotoxicity of viral vectors in damaged livers, which carry an increased risk of carcinogenesis. Thus, a benefit–risk analysis needs to be performed in each disorder before patients can be treated. In case of an oncogenic event, assessment of the causative role of a viral vector is arduous, but should include testing of the transgenic protein activity in the tumour and genomic sequencing of the tumour. In GSD1 dogs, who developed HCC following AAV gene therapy, the activity of the GSD1 deficient enzyme, G6PC, was lower in tumoural versus non-tumoural tissues suggesting that increased enzyme activity was not oncogenic in itself. However, as the integration site analyses or gene expression studies were not performed the oncogenic causality of gene therapy vector is impossible to determine.[118]

5.4.1 | LNP-mediated delivery of mRNA

The recent rapid increase of AAV-mediated clinical trials for monogenic diseases has led to increased awareness of the limitations of gene therapy mediated by viral vectors. In parallel, non-viral technologies with LNP encapsulating mRNA have reached clinical stage.[11] Few patients have been treated so far in Phase I/II clinical trials and limited information has been disclosed regarding safety and efficacy. This approach requires repeated administration due to the short half-life of mRNA.[11] However, the LNP-mRNA technology could, in theory, overcome a number of viral-mediated limitations such as absence of sustained immunogenicity enabling long-term repeated dosing, lack of genomic integration, absence of pre-existing neutralizing antibodies and thus no barrier to recruiting patients on this basis. The dose could potentially be adjusted according to the response to therapy, which is not possible with the single viral gene therapy administration. Whether LNP-mRNA could be used in the future as a bridge or lifelong therapy remains an open question.

6 | CONCLUSIONS

Liver-targeting gene therapy for inherited metabolic diseases has progressed enormously since the first clinical trials. Safer vectors, a better selection of sub-populations of patients, an increasing understanding of vector–host interactions are providing new insights to refine and improve the next generation of gene therapy vectors and strategies. In that respect, gene editing and nonviral gene therapy are holding new promises for patients and rapidly moving towards clinical translation. Persisting limitations and side effects require further innovation to be successfully overcome. The expanding community of patients having benefited from gene therapy is providing invaluable real-life information of mid- to long-term safety and efficacy.

Liver-targeting gene therapy is progressively and surely revolutionizing our therapeutic approach for patients with high unmet needs suffering from inherited metabolic diseases.

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