REVIEW

# mRNA and gene editing: Late breaking therapies in liver diseases

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## Abstract

The efficient delivery of RNA molecules to restore the expression of a missing or inadequately functioning protein in a target cell and the intentional specific modification of the host genome using engineered nucleases represent therapeutic concepts that are revolutionizing modern medicine. The initiation of several clinical trials using these approaches to treat metabolic liver disorders as well as the recently reported remarkable results obtained by patients with transthyretin amyloidosis highlight the advances in this field and show the potential of these therapies to treat these diseases safely and efficaciously. These advances have been possible due, firstly, to significant improvements made in RNA chemistry that increase its stability and prevent activation of the innate immune response and, secondly, to the development of very efficient liver-targeted RNA delivery systems. In parallel, the breakout of CRISPR/CRISPR-associated 9–based technology in the gene editing field has marked a turning point in in vivo modification of the cellular genome with therapeutic purposes, which can be based on gene supplementation, correction, or silencing. In the coming years we are likely to witness the therapeutic potential of these two strategies both separately and in combination. In this review we summarize the preclinical data obtained in animal models treated with mRNA as a therapeutic agent and discuss the different gene editing

**Abbreviations:** AATD, alpha 1-antitrypsin deficiency; AAV, adeno-associated viral vector; ABCB4, ATP binding cassette subfamily B member 4; ABE, adenine base editor; AIP, acute intermittent porphyria; ALA, aminolevulinic acid; ALT, alanine aminotransferase; ARG1, arginase 1; ATTR, transthyretin amyloidosis; C3, propionylcarnitine; Cas9, CRISPR-associated 9; CG, classic galactosemia; co, codon optimization; CRISPR, clustered regularly interspaced short palindromic repeats; CTLN2, citrullinemia Type II; CXCL10, chemokine (C-X-C motif) ligand 10; CYP7B1, cytochrome P450 oxysterol 7-α-hydroxylase; DSB, double-strand break; ERT, enzyme replacement therapy; FAH, fumarylacetoacetate hydrolase; FVIII/FVIX, factors VIII and IX; gal-1P, galactose-1 phosphate; GALT, galactose-1 phosphate uridylyltransferase; Gb3, globotriaosylceramide; GLA, galactosidase alpha; G6Pase, glucose-6-phosphatase; G6PC1, G6Pase catalytic subunit 1; GPD, glycerol-3-phosphate dehydrogenase; gRNA, guide RNA; GSD1a, glycogen storage disease 1a; h-, human; HDR, homology directed repair; HMT, hybrid mRNA technology; HNF4α, hepatocyte nuclear factor 4α; 3HP, 3-hydroxypropionate; HT1, hereditary tyrosinemia type 1; IFNγ, interferon gamma; KO, knockout; LNP, lipid nanoparticle; LLN, lipid like nanoparticles; MA, methylmalonic acid; 2MC, 2-methylcitrate; mDLNP, mRNA-loaded dendrimer LNP; MMA, methylmalonic acidemia; MPS, mucopolysaccharidosis; Mtx-LNP, LNP developed by Moderna Therapeutics; MUT, methylmalonyl-CoA mutase; NHEJ, nonhomologous end joining; NHP, nonhuman primate; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; OTCD, ornithine transcarbamylase deficiency; PA, propionic acidemia/aciduria; PBG, porphobilinogen; PBGD, porphobilinogen deaminase; PC, phosphatidylcholine; PCC, propionyl-CoA carboxylase; PCCA/PCCB, PCC alpha/beta; PCSK9, proprotein convertase subtilisin/kexin 9; PE, prime editing; PEG, polyethylene glycol; PFIC3, progressive familial intrahepatic cholestasis type 3; PKU, phenylketonuria; RBC, red blood cell; RT, reverse transcriptase; SSB, single-strand break; TT, N1,N3,N5-tris(2-aminoethyl)benzene-1,3,5-tricarboxamide; TTR, transthyretin; ZFN, zinc-finger nuclease.

strategies applied to the treatment of liver diseases, highlighting both their therapeutic efficacy as well as safety concerns.

## INTRODUCTION

The liver plays a central role in carbohydrate, lipid, and protein metabolism. It is essential in the catabolism of all types of nutrients by converting them into substances essential for the body.[1] As a result, the liver is the source for many inherited metabolic disorders that are typically autosomal recessive, caused by a single gene mutation, and occur in approximately 1 in 800 live births.[1] For several of these diseases, liver transplantation is the only curative treatment.[2] This fact supports the notion that expression of the missing protein in the parenchymal cells of the liver could potentially replace transplantation as the only curative option for these diseases.[3,4]

In recent years, in addition to conventional gene therapy based on gene supplementation using adeno-associated viral vectors (AAVs),[3–5] two different therapeutic strategies have emerged as a new class of genetic medicine with great potential to treat inherited disorders: encapsulated therapeutic mRNA and gene editing.[4–8] Several clinical development milestones were achieved with these approaches in 2021. The first patient with the rare metabolic liver disorder methylmalonic acidemia (MMA) was dosed in August 2021 with lipid nanoparticles (LNPs) carrying methylmalonyl-CoA mutase (MUT) mRNA to evaluate the safety and tolerability of the treatment.[9] In a separate study, patients suffering from transthyretin amyloidosis (ATTR) were treated with CRISPR-based gene editing technology, with extremely positive interim Phase 1 results.[10]

In this review we discuss recent experimental studies on the use of mRNA-based therapies and different gene editing strategies that are paving the way for the treatment of rare genetic metabolic diseases of high morbidity and limited therapeutic options.

## mRNA-BASED THERAPIES

The use of RNA as a therapeutic has been considered impractical for a long time due to its instability and its tendency to induce the activation of strong innate immune responses and inflammatory reactions. However, technological advances in recent decades have allowed for these obstacles to be overcome.[11] The potential of mRNA as a therapeutic is based on it being a precursor to a therapeutic protein that replaces a missing or impaired metabolic function by using the translational machinery of the target cell. Synthetic mRNA can be engineered to resemble mature mRNA molecules as they occur naturally in the cytoplasm of eukaryotic cells by including structural elements, such as the 5′ cap, the 5′ and 3′ untranslated regions, optimal Kozak sequences, and poly(A) tails.[11–13] An additional commonly used modification to improve translational yield from mRNA is codon optimization (co), achieved by replacing rare codons with more common synonymous codons, resulting in a significant increase in the expression of the therapeutic protein.[14]

However, synthetic RNA molecules are detected by different intracellular sensors, leading to the production of proinflammatory cytokines.[11] This property is of great value when mRNA molecules are used as immunogens, as perfectly demonstrated by the first approved vaccines against severe acute respiratory syndrome–coronavirus 2 infection in 2020 and 2021, but not when sustained expression of the protein is needed.[15] In 2005, the pioneering work performed by Kariko, Buckstein, and Weissman demonstrated that RNA sensors react differently when RNA nucleosides are modified. They showed that the use of specific nucleoside modifications reduces the immunogenicity of RNA molecules.[16] Altogether these modifications resulted in the development of synthetic mRNA molecules in which the duration and kinetic profile of expression of the protein product can be modulated and fine-tuned in order to obtain high levels of protein expression for periods longer than 1 week. This paved the way for the use of synthetic RNA for the treatment of different diseases including inherited and acquired diseases using the liver as a target organ.[7–9]

## mRNA DELIVERY VEHICLES

Another critically important factor for the therapeutic success of mRNA is delivery and entrance into the target cells. Because mRNA is a negatively charged macromolecule, it does not freely cross the cell membrane; hence, the levels of naked mRNA that can be taken up are low. Eukaryotic cells are capable of internalizing mRNA by scavenger receptor–mediated endocytosis, resulting in accumulation in the lysosomes; but the transfer from the lysosome to the cytosol where translation into protein can occur is minimal. To improve cellular uptake and the release of mRNA into the cytosol, encapsulation in synthetic particles of a variety of compositions has proven effective, and it additionally protects the mRNA from degradation by ribonucleases. Furthermore, these formulations can be decorated with ligands to provide the particles with the capacity to target specific cells upon systemic delivery.[17]

LNPs have been shown to very efficiently encapsulate RNA molecules and deliver them into the cellular cytoplasm. LNPs for mRNA delivery are typically comprised of four different lipids: an ionizable lipid, a neutral helper lipid, cholesterol, and a diffusible polyethylene glycol (PEG) lipid. For targeted delivery to hepatocytes, PEG lipid is essential for avoiding early capture by phagocytic cells, which is essential because liver resident macrophages, or Kupffer cells, have a great avidity for LNP-sized particles. Ionizable lipids play two functions: (1) they electrostatically bind the negatively charged RNA to facilitate cellular uptake and (2) once in the endosome, they induce endosomal escape of the mRNA/LNP lipoplex, which is finally released into the cytosol.[7-9]

The first LNP prototypes for RNA delivery caused liver damage and elicited immune responses.[18] Thus, advances in LNPs as delivery vehicles have focused on identifying LNPs with improved endosomal escape, biodegradability, and safety and tolerability profiles. One of the most frequently used LNPs in the literature for the delivery of mRNA to liver parenchymal cells was developed by Moderna Therapeutics (referred to here as Mtx-LNPs) (Figure 1). These nanoparticles have been shown to be extraordinarily efficient, reaching 100% of hepatocytes in preclinical animal models including nonhuman primates (NHPs).[7-9,14] Mtx-LNPs are internalized into hepatocytes through a process mediated by an interaction between the LDL receptor and apolipoprotein E and other opsonins. In vivo studies have shown that the ionizable lipid is fully metabolized in the liver after 6 h, indicating that Mtx-LNPs are highly degradable and rapidly cleared from the liver. Mtx-LNP mRNA nanoparticles achieve mRNA distribution primarily to the sinusoidal space at 2 h, followed by entrance into hepatocytes by 6 h, where they remain at 24 h.[14]

An alternative used for the delivery of mRNA to the liver is based on the use of a hybrid mRNA technology (HMT) delivery system, comprising two independent coadministered structures: (1) a polymer micelle for hepatocyte-specific delivery and endosomal escape and (2) an inert LNP that protects mRNA cargo from nucleases during delivery and entry into the liver
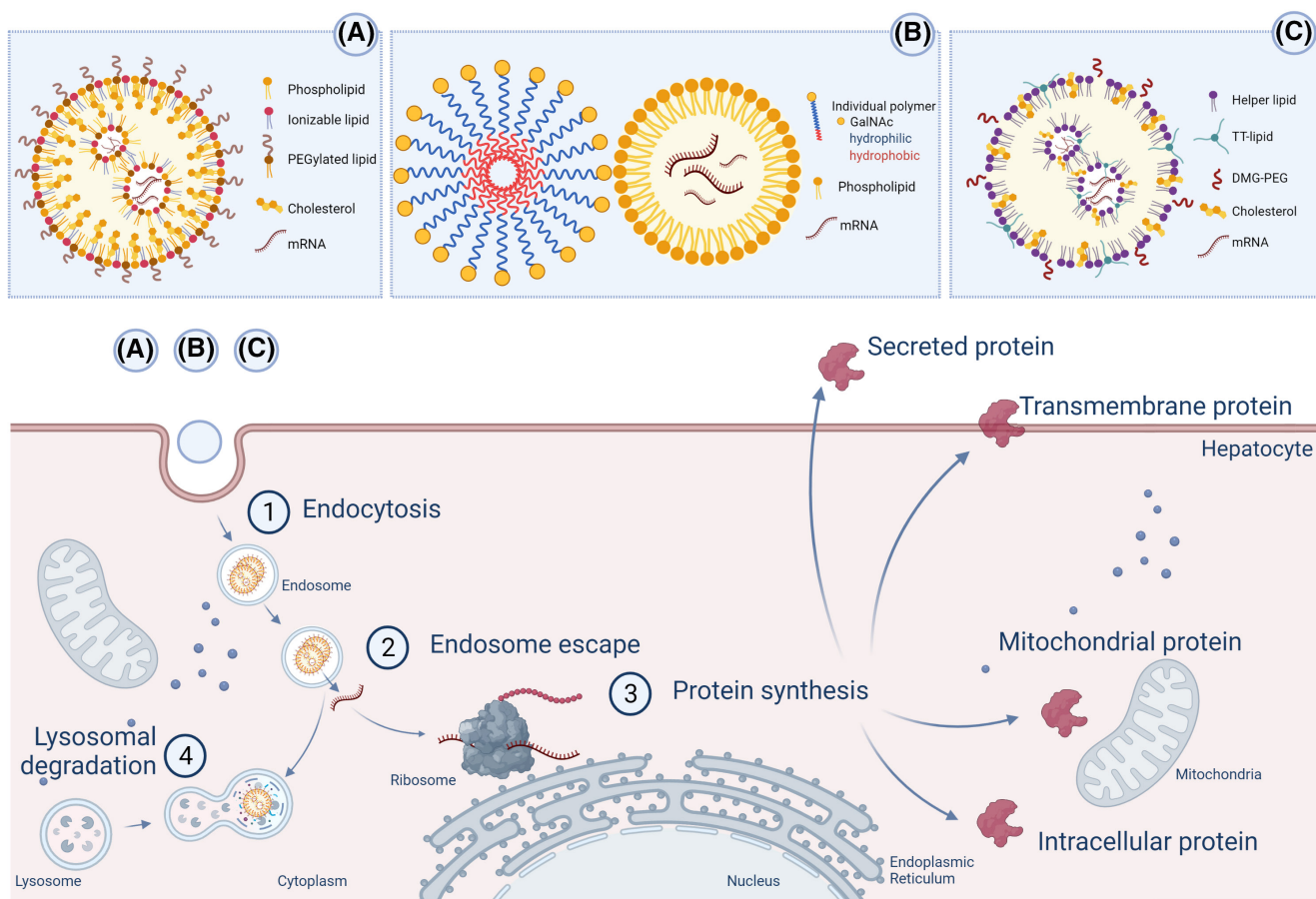


**FIGURE 1** Representation of three of the vehicles employed to deliver mRNA to the liver: (A) MTx-LNPs, (B) hybrid delivery system hybrid mRNA technology (HMT) comprising a polymer micelle for hepatocyte-specific delivery and endosomal escape and an inert LNP that protects the mRNA, (C) TT-lipid containing LNPs. A, B, and C LNPs are endocytosed by the hepatocytes; once in the cytoplasm of the cell, the mRNAs are released from endosomes to cytoplasm, where they are translated into proteins that localize in different cellular compartments according to their nature. Abbreviations: DMG-PEG, 1,2-dimyristoyl-rac-glycero-3-methoxy-PEG 2000; GalNAc, N-acetylgalactosamine

(Figure 1).[19] The polymer has three functional domains: an *N*-acetylgalactosamine molecule that binds to the asialoglycoprotein receptor that is abundantly expressed in hepatocytes, a hydrophilic polymer that maintains polymer solubility, and a polymer that mediates mRNA release from the endosome in a pH-dependent manner. The LNP is composed of (2,3-dioleoyloxy-propyl)-trimethylammonium chloride, cholesteryl hemisuccinate, cholesterol, and PEG that is passively targeted to the liver thanks to its particle size (<100 nm) and surface charge. The polymer and mRNA/LNP lipoplex each reach the liver independently, but for effective mRNA expression both components are essential.[19]

An additional type of nanoparticle used for mRNA delivery is based on *N*1,*N*3,*N*5-tris(2-aminoethyl) benzene-1,3,5-tricarboxamide (TT)–derived functionalized lipid-like nanoparticles (LLN) composed of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, cholesterol, and 1,2-dimyristoyl-rac-glycero-3-methoxy-PEG 2000 (TT-LLN) (Figure 1).[20,21]

## PRECLINICAL STUDIES WITH LNPs TARGETING THE LIVER

mRNA therapy has shown positive preclinical proof-of-concept results for several inherited and acquired diseases either affecting the liver or whose therapeutic approach is derived from using the liver as a factory for a therapeutic protein to be secreted into the circulation.

### Inherited nonhepatic diseases

Although hemophilia arises from a failure of the blood to clot properly and is not strictly a liver disease, the liver plays an essential role in the production of the missing protein responsible for the disorder. A variety of nanoparticles have been used for the delivery of mRNAs coding for coagulation factors VIII (FVIII) and IX (FIX) for the treatment of hemophilia A and B, respectively. In all cases, hepatocytes are used as protein factories that secrete the recombinant protein into circulation. Early studies performed by De Rosa et al. in 2016[22] treating FIX knockout (KO) mice with a single dose of LNP encapsulating human FIX (hFIX) mRNA at either 0.25 or 0.50 mg/kg resulted in 12-h plasma levels of hFIX protein reaching concentrations that correspond to 20% and 90% of normal physiological levels, respectively, which are well within the therapeutic range. To test the therapeutic effect, treated and untreated animals were subjected to a small incision (~1 cm) in the thoracic region at 12 h post-LNP-FIX mRNA administration, and 12 h later hematocrit levels were substantially decreased in control FIX KO mice, whereas mRNA treatment significantly protected mice from this loss. Similarly, in 2017 Ramaswamy et al. administered LNPs carrying hFIX mRNA at a 4-fold higher dose in hemophilia B mice producing hFIX serum levels within the normal physiological range (>2500 ng/mL).[23] TT-LLNs have been used for the delivery of both hFIX and hFVIII. TT-LLN hFIX mRNA at a dose of 1.1 mg/kg increased the level of hFIX in the circulation to normal physiological values (500−1500 mIU/mL).[20]

Functionalized versions of TT-LLNs (FTT-LLNs) with different types of biodegradable lipid chains have been used to treat hemophilia A mice. Intravenous injection of FTT-LLNs carrying hFVIII mRNA at a concentration of 2 mg/kg produced >140 ng/mL of the protein 12 h after injection, levels that are within the normal clinical range (−200 ng/ml).[21] Lastly, the administration of Mtx-LNPs carrying a modified version of FVIII mRNA (N6Δ2-F309S) at increasing doses from 0.2 to 2 mg/kg resulted in 200%−1000% of baseline FVIII with a single treatment.[24] FVIII expression starts rapidly, achieving peak levels at 24 h postinjection. With appropriate dosages, correction of blood clotting can be maintained for at least 7 days postinjection. However, repeated injection of the Mtx-LNPs–hFVIII mRNA resulted in the development of inhibitory antibodies against FVIII and thus long-term therapeutic efficacy could not be demonstrated in this model.[25] Due to the transient expression of the coagulation factor, the use of mRNA nanoparticles represents an attractive strategy for the prevention of bleeding in patients with hemophilia who require major surgery; however, long-term treatment might be hampered by immune reactions.

Looking at all the results as a whole, it is difficult to identify which nanoparticle is the most efficient in the delivery of mRNA to hepatocytes because different doses of mRNA were used and the method to determine FIX serum concentration varied. However, all of them achieved transiently therapeutic levels of the coagulation factor.

The use of LNP carrying coagulation factor coding mRNAs has several advantages over the use of recombinant proteins. The endogenously produced coagulation factors undergo intrinsic posttranslation modification, which potentially eliminates the increased risk of antibody formation observed after injection of the recombinant proteins. Furthermore, genetic modification of FVIII and FIX mRNAs can address the immunogenic issues as well as improve the activity of the protein to reduce the amount of protein needed to reach a therapeutic effect.[21–25] Recently, the use of emicizumab, a bispecific monoclonal antibody that bridges activated FIX and FX to restore the function of missing activated FVIII, has been shown to be highly efficient in patients with hemophilia A. However, serious thrombotic side effects have occurred in some patients, with some developing neutralizing antibody against the drug. Both undesired effects are not expected in LNP mRNA–treated patients.[25]

Alpha 1-antitrypsin deficiency (AATD) is associated with mutations in the Serpin family A member 1 gene. Patients suffering from AATD develop liver and lung pathology because the accumulation of misfolded AAT (PiZ) in the liver causes severe damage that evolves to cirrhosis, and the absence of AAT protein in the circulation is associated with the development of lung emphysema. AAT mRNA encapsulated in Mtx-LNPs was assessed by systemic delivery in the NSG-PiZ mouse model of AATD at a dose of 1 mg/kg. A trend toward higher protease inhibitory capacity, the enzymatic function of AAT, was observed in the serum of treated animals. However, the therapeutic efficacy of the treatment could not be evaluated due to the limitations of the study and the animal model.[26]

Hereditary spastic paraplegia type 5 (SPG5) is a neurodegenerative disease associated with mutations in the gene encoding cytochrome P450 oxysterol 7-$\alpha$-hydroxylase (CYP7B1), which is essential for bile acid synthesis in the liver by an alternative pathway. With an absence or deficiency of CYP7B1, patients accumulate the neurotoxic compounds oxysterols (25-hydroxycholesterol and 27-hydroxycholesterol), which are able to cross the blood–brain barrier. Intravenous administration to Cyp7b1$^{-/-}$ mice of LNPs produced by Acuitas Therapeutics and containing CYP7B1 mRNA (40 µg/mouse ≈ 2 mg/kg) led to a pronounced reduction of oxysterols in liver and serum within 2 days of treatment.[27] Most importantly, the reduction of oxysterols in serum was translated to lower levels in the brain. Four repeated injections of LNP–CYP7B1 mRNA every 5 days further reduced oxysterol levels in both organs. In SPG5 mice, contrary to patients, the accumulation of oxysterols did not lead to any obvious neurological phenotype, and thus the effect of LNP–CYP7B1 mRNA cannot be evaluated.[27]

## Inherited liver metabolic disorders

Among inherited liver diseases, the following rare metabolic disorders have been treated in preclinical studies using mRNA: MMA,[14,28] glycogen storage disease 1a (GSD1a),[29,30] acute intermittent porphyria (AIP),[31] ornithine transcarbamylase deficiency (OTCD),[19] progressive familial intrahepatic cholestasis type 3 (PFIC3),[32] classic galactosemia (CG),[33] arginase deficiency,[34] propionic acidemia,[35] citrin deficiency,[36] Fabry disease,[37] and hereditary tyrosinemia type 1 (HT1)[38] (Table 1). For most of these diseases, enzyme replacement therapy (ERT) is not an option, existing treatments are palliative or insufficient, and the only curative alternative is liver transplantation, which remains high-risk with associated long-term complications. For those indications in which ERT exists as an option, there are also several limitations associated with the treatment, such as variability in response, the development of neutralizing antibodies against the recombinant protein, infusion reactions, and differences in the glycosylation pattern of the recombinant protein versus an endogenously produced protein, which may affect its functionality and immunogenicity (Table 2). The fact that liver transplantation represents a solution for these diseases indicates that the delivery of mRNA to the liver parenchymal cells represents a promising option as an alternative to conventional ERT. This concept has been termed *intracellular ERT*.[7,8] The advantages of using mRNA to restore protein function are that the protein is produced and modified by the natural intracellular machinery, ensuring its proper folding, intracellular location, and posttranslational processing, and without modifying the genomic DNA (Table 2). For these reasons and due to the transient expression of the protein, overdosing and any associated toxicity can be better controlled.[7,8] For several of these indications, AAV carrying the therapeutic gene has been shown to be highly efficient in preventing disease progression in preclinical animal models after a single administration (reviewed in Zabaleta et al.[4] and Baruteau et al.[5]). One of the major limitations of AAV-based gene therapy is that, due to its episomal nature, vector genomes are lost as cells divide, such as hepatocytes in young animals. Furthermore, AAV readministration is not possible due to the presence of neutralizing antibodies generated after the first injection. Combination of LNP mRNA administration at an early age with AAV once liver growth has finished might represent a very attractive therapeutic strategy for inherited diseases. Importantly, AAV issues related with insertional mutagenesis or severe immune responses should be carefully evaluated and addressed, particularly when high doses are required to achieve a therapeutic effect (Table 2).[4,5]

The first inherited metabolic liver disease in which the therapeutic efficacy of LNP containing mRNA was tested was MMA, which is caused by mutations in the gene coding for MUT. Complete or partial deficiency of MUT, which mediates the final step of the oxidation of valine, isoleucine, and odd-chain fatty acids, causes a marked accumulation of toxic metabolites such as methylmalonic acid (MA). Patients with MMA suffer from growth retardation, chronic renal failure, neurologic complications, and intermittent life-threatening metabolic decompensations ultimately leading to death. In 2017, An et al. tested the therapeutic efficacy of Mtx-LNPs containing the mRNA encoding hMUT in two animal models of MMA: $Mut^{-/-}$;Tg$^{INS-MCK-Mut}$ (null Mut lethal mice that are rescued thanks to Mut expression in the muscle) and a hypomorphic MMA model $Mut^{-/-}$;Tg$^{INS-CBA-G715V}$.[14] They showed that i.v. administration of Mtx-LNPs-hMUT increased hepatic MUT activity, improved growth and survival, and reduced the concentration of toxic metabolites in plasma as well as in liver, heart, kidney, skeletal muscle, and brain for 2 days, increasing thereafter and only returning

**TABLE 1** Summary of the main findings obtained from preclinical studies using mRNA as therapeutic material for inherited metabolic liver disorders

| Disease | Short name/ OMIM | Mouse model | Delivery vehicle | Doses tested (mg/kg), frequency, and duration | Summary of main results | Safety | References |
|---|---|---|---|---|---|---|---|
| Methyl malonic acidemia | MMA 251000 | Mut$^{-/-}$;Tg$^{INS-MCK-Mut}$ | Mtx-LNP | 0.05, 0.2, 0.5 single dose | Dose-dependent reduction of MA concentration | No liver toxicity or elevation of inflammatory cytokine expression detected | [14] |
|  |  |  |  | 0.2 weekly for 5 weeks | Improved survival and ameliorated biochemical and growth abnormalities |  |  |
|  |  | Hypomorphic Mut$^{-/-}$; Tg$^{INS-CBA-G715V}$ |  | 0.5 every 2 weeks for 12 weeks | Improved survival, growth, and metabolic disturbances | Minimal decrease in serum albumin/ globulin ratio, sodium, and mild spleen alterations | [28] |
| Glycogen storage disease 1a | GSD1a 232200 | Inducible liver-specific G6Pase-α$^{-/-}$ (L-G6PC$^{-/-}$) | LNP unknown composition | 1 single dose | Reduction in fasting glycemia, liver size and steatosis, hepatic biomarkers, and triglycerides in serum; reduction in | NE | [29] |
|  |  | G6Pase-α$^{-/-}$ | Mtx-LNP | 0.1, 0.5, 2 as single dose, 0.25 in 5 doses in 8 weeks | development of liver lesions | No cytokine expression and no liver damage detected | [30] |
| Acute intermittent porphyria | AIP 176000 | C57BL/6 (Pbgd$^{tm1(neo)Uam}$) X (Pbgdtm$^{2(neo)Uam}$) | Mtx-LNP | 0.1, 0.2, 0.5 as single dose | Prevention of acute porphyria attacks; reduction of PBG and ALA urinary excretion | No major findings (only liver toxicity was evaluated) | [31] |
| Ornithine transcarbamylase deficiency | OTCD 300461 | Otc$^{spf-ash}$ | HMT | 3 single doses and 9 doses twice a week | Normalization of plasma ammonia and urinary orotic acid levels and improved survival | Minimal increases in IL-12 and CXCL10 | [19] |
| Primary familial intrahepatic cholestasis type 3 | PFIC3 602347 | BALB/c.Abcb4$^{-/-}$ | Mtx-LNP | 1–5 doses twice a week for 2 weeks | Increase PC in bile; normalization of ALT, ALP, and bile acid levels in serum; reduced fibrosis, inflammation, and ductular reaction; and improved liver weight and body weight | NE | [32] |
| Classic galactosemia | CG 230400 | GalT$^{-/-}$ | Mtx-LNP | 0.5 single dose or biweekly for 8 weeks | Significantly diminished gal-1P levels in RBCs and in liver, brain, and ovary; overcame galactose sensitivity and promoted growth in newborns | NE | [33] |
| Arginase deficiency | AD 207800 | Arg hepatic KO mice generated by treating Arg1$^{flox/flox}$ with AAVrh10-TBG-Cre | Mtx-LNP | 2 weekly or every 3 days for 2 months | Mice treated every 3 days: 100% survival with no signs of hyperammonemia or weight loss | No major findings (only liver toxicity was evaluated) | [34] |

**TABLE 1** (Continued)

| Disease | Short name/ OMIM | Mouse model | Delivery vehicle | Doses tested (mg/kg), frequency, and duration | Summary of main results | Safety | References |
|---|---|---|---|---|---|---|---|
| Propionic acidemia | PA 606054 | Hypomorphic Pcca$^{-/-}$[p.A138T] | Mtx-LNP | 0.5 or 2 every 3 weeks for 2 months<br><br>0.5 or 1 monthly for 6 months | Reduced production of toxic metabolites in a dose-dependent manner<br><br>and prevented cardiac abnormalities | Minimal increase of mitosis and inflammatory infiltrate in the liver at 2 mg/kg | [35] |
| Citrin deficiency | CTLN2 605814 | Ctrn$^{-/-}$GPD$^{-/-}$ | Mtx-LNP | 0.5 weekly for 3 weeks | Decrease of hepatic citrulline, blood ammonia, and sucrose aversion | NE | [36] |
| Fabry disease | Fabry 301500 | GLA-KO (GLA$^{tm1kul}$) | LNPs C12-200 | Single dose or monthly for 2 months | Significant reduction of Gb3 and lyso-Gb3 in kidney and heart | NE | [37] |
| | | | Mtx-LNPs | 0.2 and 0.5 every other week<br><br>0.5 mg/kg monthly for a total of 5 doses | Significant reduction of Gb3 and lyso-Gb3 in liver, kidney, spleen and heart | Safety evaluated in NHPs, no major findings reported | [38] |
| Hereditary tyrosinemia type I | HT1 276700 | Fah$^{-/-}$ | mDLNPs | 0.5 single dose | Normalization of body weight and liver function | NE | [39] |

Abbreviations: NE, not evaluated; OMIM, Online Mendelian Inheritance in Man.

to pretreatment values around Day 14. As previously described, *in situ* hybridization showed that the hMUT mRNA was localized primarily in the sinusoidal space at 2 h and then shifted to hepatocytes by 6 h, where it remained through 24 h, decreasing thereafter. In contrast, hMUT protein expression peaked at 16 h and was still detectable, although at very low levels, until day 7.[14] The pharmacokinetics of mRNA and protein expression patterns in this study has been reproduced in all the studies using Mtx-LNPs.

Next, a longer-term study was performed by the administration of six doses of Mtx-LNP–hMUT mRNA in which the first two doses were injected 28 days apart, followed by weekly injections thereafter.[24] Three different drug concentrations were used (0.1, 0.5, and 2 mg/kg), resulting in a dose-dependent and sustained reduction of MA levels in plasma. However, plasma levels of this toxic metabolite were not fully normalized and remained higher than in control animals due to the involvement of other tissues apart from the liver.[28]

GSD1a is caused by deficiency of glucose-6-phosphatase (G6Pase) encoded by the G6Pase catalytic subunit 1 (*G6PC*) gene, which provokes the accumulation of intracellular glucose-6-phosphate (G6P) and a failure to produce glucose that is associated with life-threatening hypoglycemia and long-term liver and renal complications. For example, one of the major complications is the frequent development of HCC. Inducible G6Pase-α KO mice (which possess a specific deletion of exon 3 in the G6PC gene through a cyclization recombination [CRE]-lox system) treated with LNPs encapsulating human G6PC mRNA showed normalization of blood glucose levels after fasting.[29] Furthermore, reductions in liver/body weight ratio, liver G6P, glycogen, and triglyceride concentrations were also observed. More recently, Cao et al. tested Mtx-LNPs carrying a co hG6PC mRNA expressing a G6PC with an amino acid variation to increase the enzymatic activity of the protein (S298C) in G6Pase null mice at three different doses: 0.1, 0.5, and 2 mg/kg.[30] Treated mice showed a significant improvement at all doses tested in fasting glycemia levels, reductions in liver size and steatosis, as well as reductions of hepatic biomarkers and serum triglycerides. The administration of five consecutive injections of 0.25 mg/kg Mtx-LNP mRNA over a period of 8 weeks resulted in a sustained improvement in fasting glycemia levels. However, as in earlier studies, the levels did not reach those of wild-type animals. In addition to an inability to achieve full normalization, the treatment reduced, but did not eliminate, the development of liver lesions; and 23% of treated mice and 58% of control animals developed liver tumors.[30]

AIP is a rare metabolic disorder of heme metabolism due to a deficiency in porphobilinogen deaminase (PBGD), which is characterized by the overproduction and accumulation of the neurotoxic metabolites

**TABLE 2** Advantages and disadvantages of mRNA therapy over other therapies

| Available or experimental therapies | Advantages | Disadvantages of mRNA therapy |
| --- | --- | --- |
| Dietary restriction | Proper function of the metabolic pathway | Economically more expensive |
| Enzyme replacement therapy | Natural production of the therapeutic proteins with the proper posttranslational modification; reduced immunogenicity | Limited clinical experience in the treatment of different indications |
| AAV-based gene therapy | Lower inherent risks associated with genotoxicity and immunogenicity | Multiple doses and transient transgene expression compared to one-time treatment with long-term expression |

δ-aminolevulinic acid (ALA) and porphobilinogen (PBG) when the heme synthesis pathway is activated.

Acute porphyria attacks are associated with the accumulation of ALA and PBG. Intravenous administration of an mRNA for hPBGD encapsulated in Mtx-LNPs resulted in high protein expression and enzyme activity in AIP mouse livers in less than 2 h and in full protection against acute porphyria attacks with a highly significant reduction in pain and motor neuropathy.[31] The duration of the protein expression lasted for 7–10 days, and because the mean duration of an acute attack is between 5 and 7 days, the administration of Mtx-LNP–hPBGD represented a promising option to treat acute attacks, which are currently managed by i.v. heme arginate administration that is associated with side effects such as fever, aching, malaise, and hemolysis. The safety and efficacy of the LNP mRNA treatment were also tested in larger animal models (rat, rabbit, and monkey) with encouraging results.[31] Thus, Mtx-LNP–hPBGD could potentially replace heme arginate in preventing or managing acute porphyria attacks.

OTCD results in increased ammonia levels in the bloodstream, which may lead to neurological damage, coma, and potentially death. Currently available treatments are based on a protein-restricted diet and ammonia scavengers. However, in some individuals, especially those with complete enzyme deficiency, prompt treatment does not prevent recurrent episodes of hyperammonemia and the potential development of serious complications. A recent study addressed the efficacy and tolerability of mRNA-based therapy for OTCD using the hybrid mRNA tecnology (HMT) two-nanoparticle delivery system.[19] The therapy was tested in Otc^spf-ash mice, in which OTC enzyme activity is reduced by 86% through the administration of an AAV carrying a short hairpin RNA (shRNA) targeting endogenous OTC expression. After receiving AAV-shRNA-mOTC, Otc^spf-ash animals die within 3 weeks. In this model, following a single injection of HMT carrying hOTC mRNA, OTC enzyme activity remained elevated through 10 days with 80% hepatocytes positive for hOTC protein at the peak of expression. Biweekly injection of HMT/hOTC mRNA at a dose of 3 mg/kg completely normalized plasma ammonia and urinary orotic acid levels. Importantly, all mice receiving the HMT/hOTC mRNA survived the 35-day dosing period and for at least 3 additional weeks after cessation of treatment.[19]

PFIC3 is a rare lethal autosomal recessive liver disorder caused by deficiency of the phosphatidylcholine (PC) transporter ATP binding cassette subfamily B member 4 (ABCB4)/multidrug resistance 3 (MDR3). In the absence of MDR3, PC levels in the bile are low, and the proper formation of bile mixed micelles is impaired, resulting in high biliary bile salt concentration, which damages hepatocytes and cholangiocytes. PFIC3 is characterized by persistent cholestasis that progresses to liver cirrhosis. Administration of Mtx-LNPs containing human hABCB4 mRNA to 4-week-old Abcb4^{−/−} mice at 1 mg/kg twice a week for 2 weeks resulted in the expression of functional hABCB4 protein and restored PC secretion to the bile and normalization of serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bile acid levels.[32] Furthermore, mRNA treatment reduced fibrosis by 80% as well as inflammation and ductular reaction in comparison to control animals. Additional parameters like liver weight and body weight were corrected.

CG is caused by mutations in the human galactose-1 phosphate uridylyltransferase (GALT) gene, which causes accumulation of galactose-1 phosphate (gal-1P) and deficiency in uridine diphosphogalactose. Administration of different doses of mouse GalT (mGalT) or hGALT mRNA encapsulated in Mtx-LNPs resulted in a dose-dependent increase in GalT protein expression and enzyme activity in the liver of GalT^{−/−} mice.[33] Furthermore, a single i.v. treatment of Mtx-LNP-mGalT mRNA at a dose of 0.5 mg/kg led to a decrease in gal-1P in liver and red blood cells (RBCs) within 24 h, with low levels maintained for over a week. Repeated i.v. injections of Mtx-LNP-hGALT mRNA at the same dose (biweekly for 8 weeks) significantly diminished gal-1P levels in RBCs and in liver, brain, and ovaries. Additionally, a single i.p. dose of hGALT mRNA overcame galactose sensitivity and promoted growth in GalT newborn pups.[33] Despite partially effective dietary treatment based on avoiding foods that contain lactose and galactose, the quality of life of many patients with galactosemia is very poor. Many patients suffer long-term complications, such as intellectual deficits, speech delay, impaired motor functions, and primary

ovarian insufficiency that are mainly associated with the endogenous production of gal-1P. Because mRNA therapy was able to significantly reduce gal-1P, it could potentially prevent the development of long-term pathological consequences of the disease.[33]

Arginase deficiency is a urea cycle disorder caused by mutations in arginase 1 (*ARG1*), which results in hyperargininemia and the accumulation of guanidino compounds, which cause severe neurological manifestations and progressive intellectual decline. To test the therapeutic efficacy of Mtx-LNPs encapsulating co hARG1 mRNA, a conditional arginase-deficient mouse model was used, in which the *Arg1* gene was flanked by loxP sites to circumvent the immediate lethality of arginase deficiency. For the specific elimination of arginase in the liver, mice received an AAV expressing CRE recombinase under the control of a liver-specific promoter.[34] Conditional $Arg1^{-/-}$ mice were administered with Mtx-LNP–co hArg1 at a dose of 2 mg/kg weekly or every 3 days for a period of 77 days. Weekly treatments significantly improved survival compared with control mice but did not result in long-term survival. However, mice treated every 3 days demonstrated 100% survival with no signs of hyperammonemia or weight loss. Furthermore, plasma arginine was completely normalized, and the generation of disease-related metabolites was prevented. Importantly, treated mice fully metabolized an ammonium challenge and achieved close to normal ureagenesis activity.[34]

Propionic acidemia/aciduria (PA) is an ultrarare disorder caused by deficiency of the mitochondrial enzyme propionyl-CoA carboxylase (PCC), which is composed of two subunits, alpha (PCCA) and beta (PCCB). There are two subtypes of PA: PCCA-deficient (type I) and PCCB-deficient (type II). PCC deficiency results in the accumulation of several toxic metabolites, including 2-methylcitrate (2MC), 3-hydroxypropionate (3HP) and propionylcarnitine (C3). Mtx-LNPs were used to encapsulate mRNAs encoding both hPCCA and hPCCB, and their therapeutic efficacy was tested in a hypomorphic murine model of PA ($Pcca^{-/-}$ [p.A138T]). A single administration of Mtx-LNP–h*PCCA/B* mRNAs at a dose of 1 mg/kg resulted in the hepatic expression of both proteins at the levels found in normal human liver and was associated with the normalization of plasma ammonia levels as well as a significant reduction of 2MC, 3HP, and C3 in plasma and other tissues 1 week after treatment.[35] These results represent a clear advantage over the standard of care, which is based on daily administration of carglumic acid and is unable to reduce the accumulation of 2MC, 3HP, and C3. Additionally, administration of Mtx-LNP–h*PCCA/B* mRNAs at two different doses (0.5 or 2 mg/kg administered every 3 weeks for 3 months or 0.5 or 1 mg/kg monthly for 6 months) restored functional PCC enzyme in liver and reduced the production of toxic metabolites in a dose-dependent manner. Furthermore, cardiac abnormalities were prevented by the treatment throughout the 6-month duration of the study.[35] This treatment would benefit patients with PA Types I and II.

Citrin deficiency is a recessive disorder caused by mutations in the solute carrier family 25 member 13 gene encoding the mitochondrial aspartate/glutamate transporter citrin. Citrin deficiency or citrullinemia Type II (CTLN2) causes an accumulation of citrulline and hyperammonemia, which can cause severe neurological damage. Contrary to patients with CTLN2, citrin-deficient animals failed to show any significant physiological abnormalities, including citrullinemia or hyperammonia. However, elimination of glycerol-3-phosphate dehydrogenase (GPD) in these mice (*Ctrn/mGPD* double KO) resulted in a dramatic elevation in hepatic citrulline levels upon challenge with precipitating factors such as ethanol and sucrose. In vivo efficacy of the Mtx-LNP–hCitrin mRNA was tested in *Ctrn/mGPD* double KO mice as weekly i.v. injections at 0.5 mg/kg for 3 weeks, which caused a significant reduction in hepatic citrulline compared to control mice after a sucrose challenge. Treated animals also showed a trend toward a decrease of blood ammonia and a significant reduction of sucrose aversion, a hallmark of citrin deficiency.[36]

Fabry disease is an X-linked lysosomal storage disorder cause by mutations in the galactosidase alpha (*GLA*) gene, which results in the accumulation of fatty lipids, such as globotriaosylceramide (Gb3) and its deacylated metabolite lyso-Gb3, within the lysosomes of multiple tissues as well as the vasculature and plasma. As a consequence of Gb3 and lyso-Gb3 accumulation, patients develop angiokeratomas, congestive heart failure, stroke, myocardial infarction, and end-stage renal failure, ultimately leading to death. The therapeutic efficacy of LNPs containing the cationic lipidoid C12-200 (LNPs used by De Rosa et al. for the treatment of hemophilia B[22]) carrying the mRNA encoding hGLA was tested in Fabry mice (GLA^tm1kul). These LNPs preferentially accumulated in the liver, and the highest levels of the protein were achieved there; but protein could also be detected in the spleen and kidneys at 5-fold to 6-fold lower levels. Six hours after a single administration of 1.0 mg/kg of C12-200–LNP–hGLA mRNA, 4 mg/mL of hGLA protein was detected in serum, approximately 1330-fold over the normal human physiological level. One week after the treatment, hGLA activity could still be detected in different organs like the liver, spleen, heart, and kidney. More importantly, clearances of 66% and 73% for Gb3 and lyso-Gb3, respectively, were observed in the kidney and 92% and 88% in the heart. This demonstrated better efficacy than ERT.[37] From the data reported, it is difficult to identify how much of the hGLA activity in the different organs is due to cross-correction or uptake of extracellular GLA into the lysosomes and how much is due to transfection of the tissues besides liver. Using Mtx-LNPs carrying co hGLA mRNA in which

expression was restricted to the liver showed protein distributed to different organs including the heart, kidney, and spleen. Interestingly, administration of a single dose of 0.5 mg/kg mRNA to a Fabry disease mouse model attenuated substrate accumulation in target tissues for at least 12 weeks, representing a significant benefit over ERT administered biweekly.[38]

HT1 is a rare disease caused by fumarylacetoacetate hydrolase (*FAH*) mutations, resulting in renal dysfunction, liver failure, neurological impairments, and cancer. Cheng et al. developed structure-guided rational optimization of 5A2-SC8 mRNA-loaded dendrimer LNPs (mDLNPs) carrying *Fah* mRNA.[39] The study contained very limited information about the therapeutic efficacy of these nanoparticles tested in a *Fah*-deficient mouse model (*Fah*$^{-/-}$). *Fah*$^{-/-}$ mice are protected from liver injury and death by administration of the drug 2-(2-nitro-4 -trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC). A small number of *Fah*$^{-/-}$ mice (*n* = 3) were treated i.v. with 0.35 mg/kg of mDLNP–FAH mRNA every 3 days after NTBC removal, resulting in normalization of body weight and liver function for 30 days, while control animals lost >20% of their body mass within 3 weeks after NTBC removal in the same period.[39]

## Acquired liver diseases

Acute and chronic liver injury is caused by acute or chronic viral infection or by exposure to hepatotoxic chemical compounds or toxins,[40–42] which in some patients can rapidly lead to multiple organ failure and death, with liver transplantation being the only effective treatment.[43] Rizvi et al., using a mouse model of acute liver injury from a single injection of acetaminophen (APAP) 550 mg/kg and a mouse model of chronic liver injury from the administration of a choline-deficient diet (CDD), showed that administration of LNPs carrying mRNA coding for HGF and EGF resulted in significant improvement in liver pathology.[44] In this study, mRNA produced by RNAx expressing HGF or EGF was encapsulated in LNPs composed of an ionizable cationic lipid (Acuitas Therapeutics), PC, cholesterol, and PEG lipid, an LNP that transfects virtually all hepatocytes in the liver. In mice with APAP-induced liver injury, administration of HGF and EGF mRNA LNPs at a dose of 5 μg per mouse (≈ 0.25 mg/kg) resulted in a significant reduction in liver injury 24 h later, denoted by lower transaminase levels and absence of necrotic and apoptotic cells, in comparison to control animals. In the CDD mouse model, characterized by retention of triglycerides in hepatocytes, cell dysfunction, and structural damage, administration of HGF and EGF mRNA LNPs resulted in a dramatic reduction of hepatic steatosis, injury, and necrosis as early as 2 days after treatment. The study also showed that administration of HGF mRNA LNPs was able to induce hepatocyte division

and liver regeneration. Thus, this strategy could be used to prevent liver failure in patients with acute or advanced chronic liver damage.[44]

The development of liver fibrosis, which may lead to cirrhosis and ultimately HCC, is associated with persistent liver damage due to chronic exposure to external and/or internal liver injury precursors.[45] Many inherited liver disorders, like PFIC3 and Wilson's disease, are associated with the development of liver fibrosis.[1] Multiple groups have shown that the development of liver fibrosis is associated with a down-regulation in expression of a master regulator of the hepatocyte phenotype, the hepatocyte nuclear factor 4α (HNF4α).[46] Recently, Yang et al. tested the capacity of LNPs carrying human HNF4α mRNA (formulation from Acuitas Therapeutics) to prevent the development of liver fibrosis in two mouse models: repeated administration of $CCl_4$ or a 3,5-diethoxycarbonyl-1,4-dihydrocollidine-containing diet. Administration of six doses of 2 mg/kg of LNP mRNA HNF4α every 3 days into fibrotic mice resulted in a significant reduction of transaminase and bilirubin levels as well as a marked reduction in liver fibrosis.[47] The results were comparable to those achieved by the administration of a single dose of an AAV carrying hHNF4α. Next, LNP mRNA HNF4α was tested in a mouse model with features of cirrhosis as a result of a more prolonged treatment with $CCl_4$ and in a PFIC3 mouse model, showing that the treatment inhibited the development of cirrhosis and significantly improved liver cholestasis, respectively.[47]

Altogether these data highlight the extraordinary potential of LNPs carrying mRNA for the treatment of many different types of liver diseases.

## Preclinical safety data of liver-directed LNP mRNA

In a compilation of all the data described in the different preclinical safety studies associated with the administration of mRNA-containing nanoparticles, no major issues have been found (Table 1). The analyses performed were mainly focused on liver toxicity and the activation of an inflammatory response. For liver toxicity, no transaminase (aspartate aminotransferase, ALT) elevation or major histological changes have been observed. In some studies, at the highest doses of mRNA (1 and 2 mg/kg) the presence of a mild inflammatory infiltrate and an increase in the mitotic index were reported.[35] In one particular study, a minimal to mild lymphoid depletion of the periarteriolar lymphoid sheath and red pulp cellularity was observed in the spleen.[28] Activation of the innate immune response was tested in some studies through the analysis of cytokine expression, such as interferon gamma (IFNγ), TNFα, IL-6, granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein 1, IL-12, and chemokine (C-X-C motif) ligand 10 (CXCL10).[14,19,30,35] Although in general no cytokine

expression was detected in most of the animals receiving the highest doses of mRNA, a mild increase in IFNγ, IL-6, IL-12, and/or CXCL10 was detected in some animals.[19] Surprisingly, in none of the studies was the induction of a Type I immune response evaluated, which is particularly strange taking into consideration that Toll-like receptor activation by unmodified RNA induces strong IFN Type I responses.[16] On the other hand, information about the safety of long-term administration of mRNA nanoparticles in preclinical animal models is very scarce, and this is a concern of particular importance when treating inherited diseases that require lifelong treatment.

## mRNA IN THE CLINIC FOR THE TREATMENT OF INHERITED DISEASE

The first clinical trial using mRNA formulated in LNPs posted in clinicaltrials.gov was a Phase I/II clinical trial to treat patients with OTCD (NCT03767270). The goal of the trial was to test the safety and tolerability and to evaluate the effect on metabolic disease markers and on ureagenesis of single escalating doses of LNP hOTC mRNA administered i.v. However, the trial was withdrawn prior to patient recruitment for unknown reasons. The sponsor of the trial, Translate bio, is currently recruiting patients in an mRNA-based clinical trial for the treatment of cystic fibrosis.

A month later, a second trial was posted for the treatment of patients with MMA by Moderna Therapeutics, which was also withdrawn again for unknown reasons (NCT03810690).

Currently, there are three active clinical trials of LNP mRNA, all of which are sponsored by Moderna Therapeutics. The first one, NCT04899310, is a dose escalation Phase I/II clinical trial recruiting patients with MMA due to MUT deficiency. The first patient was dosed in August 2021. In this study three doses of Mtx-LNP–hMUT mRNA (mRNA-3704) will be tested. The inclusion of an additional cohort to evaluate a fourth dose level may be considered (dose expansion phase). The drug will be administered i.v. once every 2–4 weeks, depending on participant's weight, for up to 10 doses over approximately 40 weeks.

NCT04159103 (recruiting) is a dose escalation study in 1-year-old and older patients with PA to determine the safety and tolerability of Mtx-LNP–hPCCA/B mRNA (mRNA-3927). The study is designed to characterize baseline biomarker levels followed by the assessment of safety, pharmacokinetics, and pharmacodynamics of different doses of mRNA-3927 as part of the dose optimization phase. Upon establishment of a dose with an acceptable safety and pharmacodynamic profile, additional participants will be enrolled in a dose expansion stage to allow for further characterization of the safety and pharmacodynamics of the drug.

NCT05095727 (not yet recruiting) is a dose escalation study in adult participants with GSD1a to determine the safety and tolerability of Mtx-LNP–hG6Pase mRNA (mRNA-3745) that will be i.v. administered at a single dose. Safety and tolerability will be evaluated, and hypoglycemic events will be monitored to test therapeutic efficacy during fasting challenges for up to 8 h.

## GENOME EDITING STRATEGIES FOR LIVER DISEASES

Genome editing relies on the targeted and specific modification of genomic sequences. This strategy can be used to create in vitro and in vivo animal models for preclinical experimentation, as well as to develop treatments for a variety of diseases. Here, we will review in vivo genome editing strategies that have been developed to treat inherited liver diseases.

Genome editors can be classified into two groups: nuclease-free and nuclease-guided. The former is based on long DNA sequences that are homologous to the target region and introduce the desired edit by homologous recombination.[48] This strategy is highly specific and therefore safe, but the editing efficiency is generally low. The incorporation of nucleases that generate DNA breaks (double-strand breaks [DSBs] or single-strand breaks [SSB or nicks]) in the target region has shown orders of magnitude improvement in editing efficiency but poses a greater risk of off-target effects in other genomic regions.[49] Meganucleases, transcription activator-like effector nucleases, zinc-finger nucleases (ZFN), and CRISPR/CRISPR-associated 9 (Cas9) systems have been extensively studied and described by many (Figure 1B).[6]

Several liver-directed in vivo genome editing therapies have been developed and tested preclinically due to the availability of delivery systems that can effectively target hepatocytes.[17] However, in vivo liver gene editing has been almost exclusively restricted to preclinical studies, with very few clinical trials (Table 3), until the recent publication of the exceptional clinical data using CRISPR/Cas9 to knock down the misfolded transthyretin (TTR) protein that causes the clinical manifestations of ATTR.[10] This success will likely lead to an increase of clinical trials to test liver-directed genome editing therapies in the coming years.

## INSERTION OF THE THERAPEUTIC GENE

### Specific insertion in the albumin locus

The targeted insertion of a therapeutic gene into the albumin locus takes advantage of the potent transcriptional activity triggered by the albumin promoter

in the liver, and it has shown successful therapeutic effects for genetic disorders caused by the deficiency of a secreted protein, such as hemophilia A and B or mucopolysaccharidoses (MPS). The edit consists of an in-frame insertion by homologous recombination of the therapeutic gene. Robust albumin expression in the liver drives the expression of the inserted gene, leading to therapeutic levels of the missing protein.[50,51]

The GeneRide system follows this strategy by administering a recombinant AAV carrying a therapeutic transgene flanked by 5′ and 3′ sequences with homology for the 3′ end of the albumin locus.[50] The construct is designed to induce integration of the gene upstream of the albumin stop codon preceded by a small self-cleaving protease sequence to allow the two proteins to form separately (Figure 2). Proof-of-concept studies were carried out in hemophilia B mice deficient for coagulation factor FIX. Insertion efficiency was 0.5% in both newborn and adult mice,

which was sufficient to produce therapeutic circulating FIX levels due to the potency of the albumin promoter.[51] This method theoretically avoids two major safety issues associated with the use of nucleases and AAVs for gene delivery: (1) the off-target modifications by nucleases[49] and (2) the random integration of AAV genomes leading to transactivation of oncogenic genes.[52] However, the low editing efficiency limits the indications that can stand to benefit from this therapeutic strategy. Successful preclinical efficacy data have been obtained for Crigler-Najjar syndrome[53] and MMA,[54] in addition to hemophilia B.[51]

Recently, the combination of the GeneRide system with a CRISPR/Cas9 targeting the insertion site was shown to improve editing efficiency by 20-fold to 50-fold in a Crigler-Najjar syndrome mouse model.[55]

Sangamo Therapeutics has also developed a strategy to integrate a gene of interest in the albumin locus using AAVs. In this case, homologous recombination
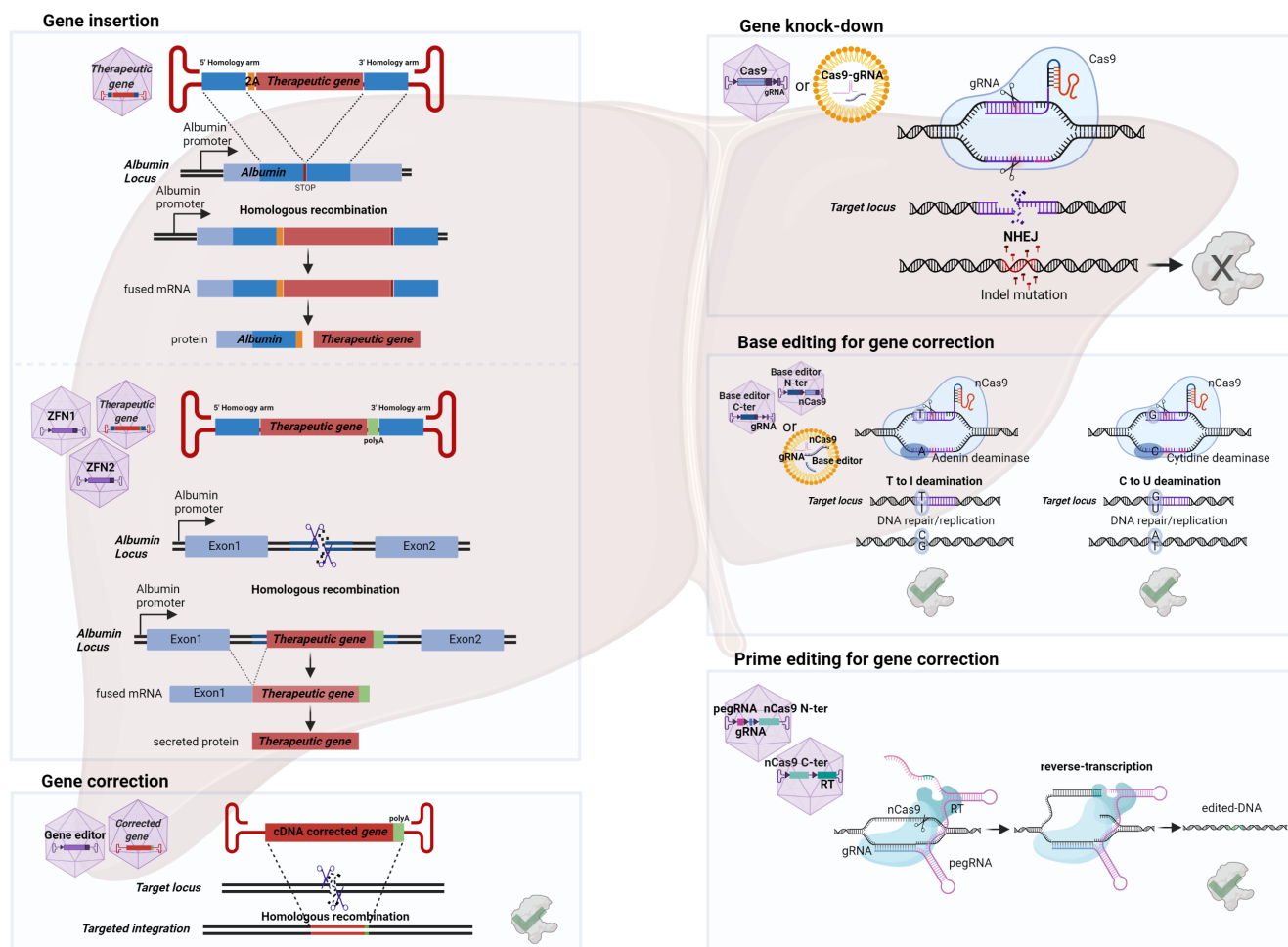


**FIGURE 2** Different gene editing approaches for the treatment of liver diseases. Gene insertion into the albumin locus by spontaneous homologous direct recombination or after the introduction of DSBs in the albumin locus using ZFN nucleases. Gene correction by homologous recombination in the target gene, DNA template provided by an AAV. CRISPR/Cas9-mediated indel formation and gene silencing after the introduction of DSBs and NHEJ. Base editing for the correction of a specific mutation for the expression of a correct version of the protein. Correction of genetic mutation by PE

occurs at intron 1 of the albumin gene to insert the open reading frame (ORF) of the therapeutic gene flanked by a splice acceptor and a polyadenylation signal (Figure 2). The edited allele is transcribed to an mRNA containing the first exon of the albumin, which encodes for a secretory signal peptide, followed by the gene of interest. The albumin gene is disrupted in the edited cells, but albumin levels in plasma are not affected due to relatively low editing efficiency.[50] The therapeutic product includes two additional AAVs encoding ZFNs targeting the albumin locus to increase the editing efficiency. Using this editing system, ~10% of mouse hepatocytes were edited and expressed the therapeutic protein. This strategy has shown therapeutic efficacy in mouse models for hemophilia A and B,[51] MPS Type II (MPSII),[56] MPSI,[57] and Fabry disease.[58] Some of the limitations of this strategy include the use of three different AAVs (with implications for manufacturing and regulatory approval), safety concerns with nucleases (although ZFNs have demonstrated low off-target cleavage), and excessive albumin disruption (potential safety concern in cases of increased editing efficiency). A similar approach using CRISPR/Cas9 instead of ZFN in a dual AAV system was shown to be efficacious for hemophilia B[59] and MPSI.[60] The advantage of CRISPR over ZFN is that only two AAVs are needed, as opposed to three in ZFN-based strategies.

## Insertion in other loci

A similar strategy to albumin locus insertion has also been described involving insertion of the therapeutic gene downstream of the apolipoprotein A1 (Apoa1) gene, which also has high expression in the liver.[61] Apoa1-targeted FAH can correct and rescue mice suffering from HT1.

Other strategies consist of homology directed repair (HDR)-mediated insertion in other loci, such as the endogenous loci or a safe harbor (e.g., ROSA26).[62–65] The efficacy of HDR was in all cases low (~1%–3% in adult mouse liver and ~10%

in newborns) but in general efficacious enough to achieve therapeutic levels of secreted proteins.[62,63] Some designs include the insertion of hyperactive forms of the transgenic protein, as is the case of the FIX Padua variant, to improve the therapeutic efficacy.[63] In a test for targeting cell autonomous diseases, such as OTCD,[64] the insertion of a cassette (promoter included) through viral vectors in newborn mice resulted in rapid and high short-term expression from the nonintegrated vector and long-term expression from the integrated form after the episomal vector gets diluted during natural liver growth.[65] Finally, this strategy is particularly successful in diseases in which the corrected hepatocytes have a selective growth advantage, such as HT1, in which edited cells have a survival advantage over unedited ones.[65,66]

## GENE CORRECTION

As mentioned, HDR can be used to modify the genome of hepatocytes in order to correct disease mutations. This strategy would ideally lead to a "scarless" edit, reverting the disease-causing mutation to a wild-type sequence. However, the disadvantage is that several editors would need to be developed for the different specific mutations appearing in different patients. Therefore, one treatment cannot be universally applied to all the patients with the same disease. Currently, the main limitation of these therapies is the low editing efficiency of HDR-based strategies. However, as mentioned, diseases in which the corrected hepatocytes are positively selected might benefit from this approach. One example is AATD in which the correction of the mutation prevents the accumulation of the mutated AAT protein and the death of the corrected hepatocytes. However, editing efficiencies remain low with the current systems.[66,67] Proof-of-concept studies have also been carried out for OCTD,[68] hemophilia B,[69] and phenylketonuria (PKU).[70] Most of these studies use dual AAV systems to deliver the gene editor (nuclease and homology

**TABLE 3** Gene editing clinical trials for liver diseases

| Disease | Editing tool | Editing strategy | Target gene | Delivery | Phase | Sponsor | Trial identifier |
|---|---|---|---|---|---|---|---|
| Hemophilia B | ZFN | Insertion in albumin locus | Albumin | AAV6 | Phase 1 | Sangamo Therapeutics | NCT02695160/ NCT04628871[a] |
| MPSI | ZFN | Insertion in albumin locus | Albumin | AAV6 | Phase 1/2 | Sangamo Therapeutics | NCT02702115/ NCT04628871[a] |
| MPSII | ZFN | Insertion in albumin locus | Albumin | AAV6 | Phase 1/2 | Sangamo Therapeutics | NCT03041324/ NCT04628871[a] |
| Hereditary ATTR | CRISPR/ Cas9 | Gene knockdown | TTR | LNP | Phase 1 | Intellia Therapeutics | NCT04601051 |

[a]Long-term follow-up.

template), although some have developed all-in-one vectors[71] or combine with nonviral delivery vehicles, such as LNPs.[72]

## CRISPR/Cas9-MEDIATED GENE KNOCKDOWN

Nuclease-based gene editors create DSBs in the target genome, with the breaks being predominantly fixed by nonhomologous end joining (NHEJ). NHEJ repair introduces random insertions and deletions (indels) in the repaired sequence. This mechanism has been used to introduce errors in coding regions leading to protein knockdown by nonsense-mediated decay of the edited mRNA.[73] This strategy has been used for substrate reduction therapies for diseases in which the deficiency of an enzyme leads to accumulation of toxic metabolites. Inhibition of enzymes that produce the toxic metabolite upstream of the catalytic step that is impaired can achieve therapeutic benefit. This is the case of primary hyperoxaluria, which is characterized by the accumulation of oxalate in the kidneys. The CRISPR/Cas9-mediated inhibition of enzymes upstream in the metabolic pathway for oxalate synthesis led to a therapeutic decrease of oxalate levels produced in the liver.[74,75] This same strategy has also been used to regulate cholesterol levels.[76,77] Finally, knocking down mutated proteins that accumulate aberrantly has also shown therapeutic efficacy. Recently, Intellia Therapeutics has reported the results of the first-in-human application of this strategy to treat ATTR. ATTR is caused by mutations in the TTR protein that accumulates in tissues forming amyloid fibrils. Gillmore et al. report a strategy based on LNPs that carry the mRNA of Cas9 and the single-guide RNA (sgRNA) that targets TTR. This technology showed dose-dependent reduction of up to 96% of circulating TTR in patients with ATTR.[10] These exceptional results offer a great precedent for LNP-delivered gene editing therapeutics.

## BASE EDITING

The versatility of the CRISPR/Cas9 system has allowed for alternative applications to be designed upon modification of the core components, such as highly efficient base editing in vivo.[78] Base editing involves a Cas9 nuclease engineered to create a nick at a target DNA site where a single-strand DNA–specific cytidine deaminase or transfer RNA adenosine deaminase that is conjugated to the Cas9 catalytically changes the host DNA sequence by converting C●G base pairs to T●A base pairs or converting A●T base pairs to G●C base pairs, respectively. These are called cytosine base editors and adenine base editors (ABEs), respectively (Figure 2).

Base editing strategies have been employed for the treatment of liver disorders. Several proof-of-concept mouse studies have been carried out to edit cholesterol-related genes[79] and the genes that cause PKU[80] and hereditary HT1.[81] Due to the large size of base editors, adenovirus, dual AAV systems, and hydrodynamic tail vein injection have been used as delivery methods for the initial proof-of-concept mouse studies. Mouse or human proprotein convertase subtilisin/kexin 9 (PCSK9) gene has been targeted in two of these studies, aiming to create an early stop codon and disrupt the ORF in order to reduce cholesterol levels.[79,82] As mentioned, PCSK9 has also been knocked down by NHEJ strategies; however, base editing is potentially safer because of the specific modification of the genome compared to random repairs. The correction of disease-causing mutations by base editors was found to be therapeutically efficacious in mouse models for HT1 and PKU. In the PKU study, an intein system was used to deliver the large base editor in two AAVs (Figure 2).[83]

The advantage of genome editing over other forms of gene therapy is that long-lasting expression is not required; hence, a transient expression of the editor is sufficient and preferred to avoid off-target effects and immunogenicity. The therapeutic base editing of the PKU mouse model was proven to be efficacious in vivo when using LNPs that carry the RNA components of the base editor. Recently, promising results have been reported by two groups that targeted PCSK9 in NHPs[84,85] using LNPs to deliver ABE RNA components. Both strategies consisted of targeting splice sites to generate aberrant splicing variants and knock down the PCSK9 protein. Even though both treatments targeted the same region of the gene with a similar delivery method, the editing efficiency differed between the two studies, with >60% in one study[84] and ~30% in the other.[85] The studies used two differently evolved ABE systems, which might explain the large differences in efficacy.

Overall, the main advantage of base editors is the potential to achieve highly efficient gene correction, in contrast to HDR strategies. However, an extensive characterization of the guide RNA (gRNA) needs to be done to avoid bystander base editing in the target region.

## FUTURE STRATEGIES: PRIME EDITING

Prime editing (PE) was developed in 2019 as a system that combines the CRISPR/Cas9 system with a reverse transcriptase (RT).[86] The Cas9 is fused to an RT enzyme, and the gRNA is fused to a template RNA used by the RT to introduce edits, called prime editing guide RNA (pegRNA). The Cas9 gRNA brings the PE complex to the target site and generates a nick that allows for the RT to synthesize a complementary DNA sequence containing the desired modification

(Figure 2). This system allows for virtually any desired edit, and the efficiency has been found to be high in cells. Additionally, the efficiency and editing ability have been expanded upon in subsequent publications.[87,88]

PE has been used to correct disease-causing mutations in vitro and *ex vivo*.[89–91] However, the large size of the editor has limited its application in vivo. Several PE delivery systems have been developed and tested in mouse models: dual AAVs,[92,93] fully gutted adenovirus,[94] or hydrodynamic tail vein injection.[89,95] Some groups have corrected the *Fah* allele in HT1 mice using dual AAVs[92] or hydrodynamic tail vein injection of plasmids encoding a PE.[89] This same group created a triple AAV treatment to correct a genetic eye disease using PE, which could potentially be applied to target the liver.[89] A dual AAV system has also been used to correct the PiZ allele in an AATD model by adapting the PE system to *Staphylococcus aureus* Cas9 (SaCas9) to reduce the size of the editor and fit the whole system (including pegRNA and sgRNA) into two AAV vectors.[92]

PE is a promising strategy to correct disease-causing mutations in a scarless way but with higher efficiency than HDR. However, further research needs to be performed to improve delivery methods in vivo.

## SAFETY OF CRISPR/Cas9 GENE EDITING

As reviewed in this section, CRISPR/Cas9 systems have shown the potential and the adaptability to efficaciously treat genetic liver disorders.[86] However, the safety of this therapy is under evaluation in preclinical studies and in the limited number of clinical trials that are being performed. The two main concerns regarding treatments involving exogenous nucleases are off-target DNA editing and an immune response to the Cas9 protein.

Evaluation of off-target activity has been extensively studied in vitro or *ex vivo* using different methods such as genome-wide unbiased identification of DSBs enabled by sequencing (GUIDE-seq)[96] and its variations such as target enriched GUIDE-seq that enriches the hits,[97] and others such as circularization for high-throughput analysis of nuclease genome-wide effects by sequencing,[98] circularization for in vitro reporting of cleavage effects by sequencing,[99] oligonucleotide enrichment and sequencing, and selective enrichment and identification of adapter-tagged DNA by sequencing (SITE-seq).[100] The main limitation of these methods is that they do not necessarily predict the in vivo off-target effects. Recently, chromosomal aberrations analysis by single targeted linker-mediated PCR sequencing was developed to identify chromosomal rearrangements produced after the DSB caused by Cas9 in on-target and off-target

regions.[101] Finally, an example of other deep sequencing methods that could be adopted for in vivo analysis is whole-genome sequencing, which could be applied as an unbiased method to assess off-target events. However, the low frequency of some off-targets might not be detected without enrichment.

Another limitation in the analysis of the off-targets is that they are gRNA-specific and, therefore, species-specific in many cases. Therefore, identification of off-targets of clinically relevant gene editing products is limited to in vitro models, humanized mice, and NHP models (with limitations). Gillmore et al. used primary human hepatocytes to assess off-targets of their LNP gene editing product before clinical testing. They combined predictions of off-targets with in vitro GUIDE-seq and SITE-seq methods to identify the off-targets of their product.[10] There has not been strong evidence that suggests off-target modifications in vivo so far. A strategy to minimize unwanted modifications is to use high-fidelity Cas9 nucleases that show improved specificity and precision[102] and an eventually modified version of these Cas9 including nickase Cas9, which generates SSBs that are mainly repaired by high-fidelity SSB repair pathways.[103] Thus, only in combination with a pair of gRNAs targeting opposite strands of the target locus a short distance apart may they generate controlled on-target editing, decreasing the likelihood of off-target events.[104]

Besides off-target activity, gene-editing based on CRISPR/Cas9 may be immunogenic. In fact, the CRISPR/Cas9 systems used with therapeutic purposes derive from bacteria that are in contact with humans, such as *Streptococcus pyogenes* (Sp) and *Staphylococcus aureus*. Evaluation of humoral and cellular immunogenicity against Cas9 showed that a high percentage of healthy humans react against SpCas9 and SaCas9.[105] Therefore, immune reactions against CRISPR/Cas9-based therapies represent a safety concern that could lead to acute or long-term toxicity and reduction of therapeutic efficacy. Apart from preexisting immunity, humoral and cellular immune responses are elicited in some mouse models after treatment with AAVs expressing CRISPR/Cas9,[106] which could also lead to unwanted long-term effects. In this sense, the delivery method plays an important role, with delivery systems that provide transient Cas9 expression and low inflammatory responses preferred, such as nanoparticles.[107]

## SUMMARY

In summary, the use of mRNA as a therapeutic molecule and the modification of the patient genome by CRISPR/Cas9-based technologies have revolutionized the current landscape of genetic medicine. The astonishing and rapid advances made in both

technologies in the last decade have led to the first clinical applications only just recently. The rational modification of mRNA molecules to achieve high protein expression in the target cell without being detected by the immune system as well as the improvement in the delivery systems have been crucial to its success. Additionally, in the case of CRISPR/ Cas9, the engineering of its components has led to incredible versatility of gene therapy applications, from therapeutic gene insertion to precise gene correction and targeted knockdown. With the development of these two technologies, the wealth of alternative approaches has increased immensely to treat diseases with very limited therapeutic options, which is the case for most inherited metabolic liver disorders.

As reviewed here, both mRNA and gene editing have proven to be safe and highly efficacious in preclinical models. However, as with gene therapy mediated by recombinant AAV, we will only have a better grasp on the full limitations and problems following clinical trial data, and these trials have only just begun.

Nevertheless, more and more clinical experience will be achieved in the coming years using genome editing and mRNA therapy, which will certainly establish the value of these promising technologies in the realm of improving the lives of patients.

## CONFLICT OF INTEREST

Dr. Gonzalez-Aseguinolaza owns stock in and is the chief scientific officer and cofounder of Vivet Therapeutics. Dr. Weber owns stock in and is employed by Vivet Therapeutics.

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