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Gene Therapies in the Fight against Liver Disease: A Comprehensive Review

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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Review Article

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ABSTRACT

Introduction: The field of hepatology is rapidly evolving, with new treatments being investigated using gene therapy. Gene therapy involves using a patient's genetic code and altering it to create a desired phenotype/genotype.
Methods: Within this emerging domain, there is a multitude of delivery mediums used to alter genes. It is essential to understand the mechanisms of genetic therapies and the risks and benefits

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associated with them. This review aims to break down the mechanisms of action of gene therapies, describe current research conducted, and discuss future implications for the field of hepatology.
Discussion and Conclusion: Different methods of gene therapy include gene editing, gene silencing, gene transfer, and mRNA therapy. These genetic modulations are achieved through several modalities. Examples include zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), cluster-regulated interspaced short palindromic repeats with associated cast 9 proteins (CRISPR/Cas9), SiRNA particles, antisense oligonucleotides (ASO), and synthetically engineered mRNA. Initially, the method for gene therapy included injecting genetic code, usually in a vector, to create phenotypic production in gene-deficient states. Recently, gene therapy has included more precise editing of the genome with technologies such as CRISPR-Cas9 and silencing of pathogenic genes.

Keywords: Gene therapies; vectors; CRISPR-Cas9; TALENs; ZFNs; ASOs; mRNA therapy; liver disease.

1. INTRODUCTION

The field of hepatology has drastically changed over the last two decades with rapid growth in treatment options for chronic liver diseases such as hepatitis C, and new pathogenic processes being identified including the genetic basis for several diseases. The field continues to push the envelope of treatments with gene therapy which is the concept of using a patient's genetic code and altering it to create a desired phenotype/genotype. Starting with vectors: there are viral vectors as well as nonviral vectors to help deliver genetic code into cells. There are methods of delivering naked genetic material using hemodynamic injections. Delivery vesicles can also be modified to target liver-specific cells [7,11]. Gene editing, gene silencing, gene transfer, and mRNA therapy are different methods of achieving the desired phenotype/genotype.

2. METHODS

This research has focused mostly on gene editing with the use of zinc finger nucleases (ZFNs), transcription activator like effector nucleases (TALENs), and cluster regulated interspaced short palindromic repeats with associated cast 9 proteins (CRISPR/Cas9). ZFNs are nucleases that use multiple base pairs to bind to specific parts of DNA and cause double-strand breaks, where these breaks occur, DNA can be introduced [13]. mRNA therapy is a treatment that has gained a lot of traction recently as a gene-modifying therapy. mRNA therapy in a simplified manner, is synthetically engineered mRNA which is injected into cells to allow for the replication of specific proteins. The goals of these therapies in hepatology are broad, ranging from treatment for hepatitis B virus (HBV), and rare genetic liver diseases, to providing a cure for hepatocellular carcinoma (HCC). This paper aims to simplify complex but pertinent gene therapies that are currently being studied and provide an overview of current trials. A fundamental understanding of these future therapies is important for clinicians and researchers involved in taking care of liver diseases.

3. DISCUSSION

3.1 Delivery Vectors

3.1.1 Viral delivery vectors

Adenoviral vector: Adenoviruses are non-enveloped double-stranded DNA vectors that have been used in the field of oncology [1]. Their maximum packaging capacity is anywhere from 36-37 kb [1,8]. The advantage of using adenovirus vectors is their efficient transduction into liver cells [1]. The disadvantages to adenoviral vectors include the possible elicitation of a strong immune response, the presence of serotype-dependent pre-existing immunity, and the development of acute inflammatory responses [1,8]. The utility of adenoviral vectors has been discussed in several studies examining the safety and efficacy of such a medium. One study displayed successful lacZ gene (gene that creates β-galactosidase which cleaves lactose, a disaccharide, into glucose and galactose) transfer via adenoviral vectors to normal and cirrhotic livers in a mouse model [2-5]. Histochemical evaluation revealed marked transgene expression even in fulminant hepatitis mouse livers without significant differences in cellular or humoral immune response among normal, cirrhotic, and hepatitis mouse livers [14]. A similar study portrayed the therapeutic

potential of adenoviral vectors, specifically in the role against hepatocellular carcinoma [9,10]. In-vivo and In-vitro suppression of hepatocellular carcinoma was achieved through delivery of adenoviral vector Ad-ECRG2 [15]. Esophageal cancer-related gene 2 is critical in carcinogenesis. Ad-ECRG2 successfully suppressed the invasion and adhesion of cancer cells while altering the expression of multiple key cancer-related molecules [15].

Adeno-associated virus derived vector: Adeno-associated viruses (AAV) are non-enveloped single-stranded DNA vectors that can act as a delivery system for genetic material into cells [1]. Utilization of this type of viral vector in theory would require serotype testing prior to initiation of treatment to determine efficacy. There has been a proof-of-concept study completed using AAV's with the induction of hepatitis B virus (HBV). The study implemented AAV8 to carry HBV genotype D into mouse cells to stimulate a chronic infection. A similar study was completed in rhesus monkeys, a species that is not naturally capable of being infected with HBV, where the monkeys were given the HBV specific receptor hNTCP carried in AAV8. These Rhesus monkeys were then infected with HBV and showed viral replication up to 24 weeks after inoculation [6]. AAV vectors are currently being utilized in several clinical trials and are well tolerated. This vector has been utilized in Phase I studies targeting Hemophilia B, [16-26] Hemophilia A, [27-32] Ornithine Transcarbamylase (OTC) deficiency, [33-36] Phenylketonuria, [37,38] Acute Intermittent Porphyria (AIP),[39] Methylmalonic Acidemia, [40] Familial Hypercholesterolemia, [41] Fabry Disease, [42-44] Mucopolysaccharide Syndrome (MPS) I, MPS II, MPS IV, [21,45-47] Wilsons Disease, [48] and Crigler Najjar [49,50]. Phase II studies are ongoing targeting several of these pathological conditions.

Lipid Nanoparticles: Lipid nanoparticles are manufactured similarly to cell membranes to allow for passage through the membrane. They are composed of amphiphilic lipids creating a micelle with genetic material trapped on the inside with a hydrophilic layer, and outside coated hydrophobic layer. The bio-similarity to cell membranes gives the lipid nanoparticle low toxicity, low immunogenicity, and structural flexibility [8]. Other advantages that lipid nanoparticles provide are their ability to be mass produced and their inherent biodegradability [8]. The disadvantage of using lipid nanoparticles is their lack of specificity for cells. This problem has been combated by binding N-acetylgalactosamine (GalNAc) clusters to the lipid nanoparticles to help target cells more specifically [8]. Another method to help specificity of lipid nanoparticles is the use of polyethelyne glycol (PEG). This was shown in a medication approved by the FDA in 2018 for TTR-type familial amyloid polyneuropathy. The lipid nanoparticle used a PEG siRNA system that targeted transthyretin [11]. The lipid nanoparticle is taken up via endocytosis by the low-density lipoprotein receptor on cell membranes. Lipid nanoparticle therapies are being utilized in clinical trials utilizing mRNA therapy and the CRISPR/Cas9 gene editing technique.

Gene Editing: Gene editing is the process of targeting specific sections of DNA and altering or replacing those sequences for the desired product. There are two main classes of editing: nuclease-guided and nuclease-free editing [12]. The three main nucleases used in editing are zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeats with associated Cas9 protein (CRISPR/Cas9) [1,8]. These techniques are combined with either non-homologous end-joining, or homology-directed repair. These repair mechanisms allow for insertion or deletion of desired genetic code. The most common target is the albumin locus in the liver, given its high transcriptional capabilities [12]. Non-homologous end-joining allows for insertion or deletion of variable lengths, which tends to cause frameshift mutations and can lead to gene knockout [56,55]. RNA-guided nuclease editing tends to have higher efficiency of integration [57]. Nuclease-facilitated editing also has the potential for off-target effects secondary to Cas9. Due to this problem, nuclease-free editing was developed based on homology-directed repair but it is less efficient [58].

Zinc-Finger Nucleases: Zinc-finger nucleases (ZFNs) are nucleases that use multiple base pairs to bind to specific parts of DNA and cause double-strand breaks [13]. ZFNs are made up of 30 amino acids bound to a molecule of zinc [13,59]. There are five amino acids that do not fold around the zinc molecule, which act as a link between the zinc-fingers [60]. The amino acids bind to 3-4 complementary nucleotides of dsDNA that they have specificity towards [59]. The ZFN will bind to one strand of DNA using multiple base pairs, with a cleavage domain (FokI nuclease) at the end, while another ZFN group of amino acids will attach to the other strand of

dsDNA, creating a double-strand break (Fig. 1). The locations of the breaks can then be targeted with newly introduced genetic code. There have been two phase II trials using ZFNs directed at the albumin locus of hepatocytes targeting Alpha-L-Iduronidase and Iduronate 2 sulfatase in MPSI and MPSII, respectively [51-53]. Both of these trials have been rolled to long-term follow-up studies [61,62]. Previous trials also have used adeno-associated viral vectors modified with ZFN to target Factor IX at the albumin locus in Hemophilia B [17,21].

Transcription Activator-Like Effector Nucleases: Transcription activator-like effector nucleases (TALENs) are similar to ZFNs by the fact that they use a FokI nuclease domain to create the double-strand breaks (Fig. 2). TALE proteins occur naturally in the bacteria xanthomonads, and have been shown to attach FokI domain to alter the genes of plants [63]. This domain attached to the FokI domain is the transcription activator-like effector repeat domain. This set of protein codes is flanked by a C-terminal domain and an N-terminal domain, which allows for the replacement of genetic code with complementary pairing to attach at the C-terminus and N-terminus. These terminus amino acids allow for more cost-effective and efficient production over ZFNs. This also allows TALENs to be specific on where the double-strand break will occur. Similar to ZFNs, two TALENs are needed to cause the double-strand DNA break.[56] This gene therapy has been shown to be effective in mouse liver models of chronic HBV, where markers of viral replication were inhibited after the introduction of TALENs targeting the four HBV-specific sites within the viral genome [64].

CRISPR/Cas9: The CRISPR/Cas9 system is the immunologic system of prokaryotes. The CRISPR sequence is a strand of DNA that encodes protein production for the Cas9 system. The Cas9 system is a set of proteins that break down bacteriophage DNA upstream from the CRISPR sequence. The CRISPR sequence has a region of protospacer adjacent motifs (PAMs) on both sides (Fig. 3) [65]. The PAM region has an allosteric effect that helps the Cas9 region break down DNA [65]. All together the bacteria will detect foreign DNA, and break it down using Cas9 proteins. These broken DNA pieces attach to the PAM region and are sent to incorporate into the CRISPR sequence. The CRISPR sequence will then replicate and send RNA to the Cas9 region to create protein complexes specific for that broken DNA (Fig. 4). This allows the PAM to bind specific DNA and the Cas 9 proteins to efficiently break down that specific foreign DNA quickly. This is currently the favored genetic therapy technique [8]. There has been recent research using CRISPR/Cas9 via AAVs with anti-HBV DNA. There have been new discoveries using AAVs to avoid second-strand synthesis, which decreases transgene expression. This is done by mutating the terminal resolution site (trs) site on the AAVs, allowing for hairpin loops to form reducing its packaging size, and are called self-complementary AAVs (scAAVs) [66]. With this smaller packaging size the scAAVs studies were able to use a Staph Aureus Cas9 with multiple cccDNA targets through RNAi targeting HBV and Argonaut2 in mice with chronic HBV [67]. These studies showed a significant decline in HBV replication in both cultures and mice [68]. There is an active phase I clinical trial using lipid nanoparticles as a vector for the CRISPR/CAS9 gene editing technology targeting the transthyretin protein, present predominantly in the liver, which is misfolded and accumulates in this condition [69].

Gene Silencing: Gene silencing identifies an unwanted DNA sequence and effectively stops cells from replicating this sequence, which can be done by targeting mRNA using small interfering RNA (siRNA) or antisense oligonucleotides (ASO). siRNA and ASOs are similar in concept; they both use Watson-Crick base pairing to attach to host mRNA and suppress replication [72].

Small Interfering RNA: siRNA is a double-stranded RNA that causes “RNA interference,” which is double-stranded RNA binding complementary mRNA and silencing the gene [70-72]. This process is done by cleavage of the double-stranded RNA by RNase III-like enzyme. The more stable siRNA binds to the mRNA and activates a protein called RNA-induced silencing complex (RISC). This RISC causes the mRNA to be cleaved and effectively silenced [73]. The use of siRNA with a naked delivery system was discussed in a previous section, but siRNA can be delivered using any traditional method weighing the pros and cons, i.e. nonviral vectors, viral vectors, or naked. Using the nonviral mechanisms has its main limitation of having to re-administer doses after a period time, it is not a sustained response [1]. Specifically for the delivery of siRNA to hepatocytes, recent development of a polymer-siRNA conjugate called Dynamic PolyConjugates (DPC) has shown promise.

The study demonstrated effective knockout of apolipoprotein B and proliferator-activated receptor alpha [74]. GalNAc and siRNA have been a focus of research with FDA-approved drugs Givosiran for acute hepatic porphyria, [75,76] and Lumasiran for the treatment of primary hyperoxaluria type 1. [77] There are currently ongoing studies looking at GalNAc-modified siRNA for HBV, NASH, GSD1a, and hereditary hemochromatosis [8,78-81]. There is currently a siRNA-LNP that has completed a phase III trial for familial hypercholesterolemia targeting PCSK9 and apoB [54,82,83]. This vector method is also being explored for chronic hepatitis B with a recently completed Phase II clinical trial [84]. There is also a completed phase II trial involving the development of a siRNA targeting polo-like kinase 1 (PLK 1), which is overexpressed in hepatocellular carcinoma. The specific targets for this therapy are oncogene MYC and polo-like kinase-1 [85].

Antisense Oligonucleotides: There are multiple types of ASOs that are all single-stranded antisense molecules. The original ASOs consisted of a phosphodiester backbone and were called unmodified ASOs. Recent investigations have been using different types of modifications to ASOs with different benefits. The list of modified ASOs include: phosphorothioates (PS), phosphorodiamidate morpholinos (PMO), peptic nucleic acids (PNA), locked nucleic acids (LNA), 2'-O-methyls, 2'-O-methoxyethyls, 2' fluoros, 5' methylcytosines, and G-clamps [86]. PS have a modification to the phosphate group, allowing for enzymatic stability. PMOs and PNAs have sugar phosphate modifications, PMOs have the benefit of improved solubility and binding affinity; PNAs have increased enzymatic stability, better binding affinity, and do not activate the immune system. LNAs, 2'-O-methyls, 2'-O-methoxyethyls, and 2'fluoros have sugar modification. LNAs have stability and high binding affinity. 2'-O-methyls and 2'-O-methoxyethyls have high binding affinity, stability, and decreased immune reactions [87]. 2'fluoros have high binding affinity. 5'methylcytosine and G-clamp use nucleobase modification. Both 5'methylcytosine and G-clamp have high binding affinity, and 5'methylcytosine has no immune reaction [87]. There is another genetic treatment in progress for NASH using GalNAc-modified ASOs [88]. This treatment seeks to target serine/threonine protein kinase or fat-specific protein 27. ASO therapy has already been approved for the treatment of homozygous familial hyperlipidemia with the drug Mipomersen [88]. Other trials utilizing antisense oligonucleotides have been studied in transthyretin TTR amyloidosis. There is an ongoing phase III clinical trial of a ligand-conjugated antisense oligonucleotide against the TTR protein [89].

mRNA Therapy: The rationale for mRNA therapy centers on synthetically engineered mRNA injected into cells to allow for replication of specific proteins. Though oversimplified, this allows an understanding of the concept for a more in-depth discussion of the process and future implications. mRNA therapy creates a transient effect in protein production. This has a benefit of allowing for dose control but requires the need for repeat dosing for a sustained effect [1]. The synthetic mRNA structurally is a 5' cap, 5' to 3' untranslated regions, kozak sequences, and poly-A tails [12]. This structure is designed to resemble a naturally occurring mature mRNA found within the cytoplasm. It was discovered that certain RNA nucleoside modifications led to better control of duration, kinetic profile and decreased immunogenicity [90]. This was a major breakthrough in the development of this method. The modified mRNA is negatively charged and is unable to freely cross the cell membrane into cells. This has led to the use of multiple methods to impregnate cells with synthetic mRNA, similar to other genetic editing mechanisms. Eukaryotic cells have the ability to actively transport these negatively charged particles across the cell membrane with the use of endocytosis [12].

Genetic therapies seem to be on the forefront of the hepatology world. Hundreds of animal studies and clinical trials are currently underway for a variety of liver diseases. Most of these trials are in the starting phase one and phase two, but there are also gene therapies that are currently FDA-approved and being used in clinical practice. Adenoviral vector gene transfer is well described in previous clinical trials and in the literature. AAV therapy has promising results in treatment for Hemophilia A. The liver is the site of production for clotting factors (liver parenchymal cells produce coagulation factors involving generation of fibrin clot, except factor VIII which was synthesized by hepatic and extrahepatic endothelial cells) and can serve as a site for AAV therapy [92,93]. A multiyear follow-up study was conducted after a single administration of AAV5 genetic transfer normal VIII factor which resulted in a sustained, clinically relevant benefit in 15 participants [94]. In seven participants, bleeding events decreased from a

mean of 16.3 events per year to 0.7 events per year at the end of year 3, a 96% reduction [94]. In six participants bleeding events decreased from a mean of 12.2 events per year to 1.2 events per year at the end of year 2, a 92% decrease [94]. A phase III trial was conducted with valoctocogene roxaparvovec, a B-domain-deleted factor VIII coding sequence with AAV genetic transfer [29,95]. In this study 134 men were included and the mean annualized treated bleeding rate decreased by 84.5% (p=<0.001) from baseline [95]. During the time of the study there were no serious adverse events related to the treatment [95]. AAV therapy has also shown to be effective in the treatment of Hemophilia B. A phase III trial was conducted which administered one infusion of AAV5 vector containing a Padua factor IX variant [16]. It was found that annualized bleeding rate decreased from 4.19 to 1.51 during months 7 through 18 after treatment (p=<0.001). It was concluded that etranacogene dezaparvovec gene therapy was superior to standard prophylactic factor IX, and showed a favorable safety profile [96]. Another phase I clinical trial targeting porphobilinogen deaminase haploinsufficiency, an enzyme deficient in AIP showed promising results for AAV gene therapy. In this clinical trial two patients had a positive clinical outcome that resulted in the cessation of hematin treatment [97]. AAV genetic transfer therapy is promising for pathologies in which a genetic derangement results in decreased activity of an essential enzyme. However, the division of cells over time causes vector dilution and repeat vector therapy that may be ineffective due to the immune response of the patient [98-101].

Specifically, for A1AT deficiency, there are promising SiRNA trials currently in phase two. Fazirsiran, an siRNA, has shown promising results in an active phase II clinical trial where results showed a significant reduction in the alpha 1 antitrypsin Z mutant protein [102].

Current hepatitis B trials seem to be focused on mostly the use of siRNA targeting HBV RNA, with multiple trials in phase 1. These studies work to decrease the expression of hepatitis B surface antigen messenger mRNA in liver hepatocytes. One study used DCR-HBVS which is a synthetic RNAi which is conjuges to GalNAc ligands [81]. Another study utilized AB-729, a siRNA inhibitor of HBV, in combination with vebicorvir, a novel core inhibitor [103].

There are currently two NASH trials using ASO. One trial in phase I, using ASOs to target the PNPLA3 gene. This mouse trial has shown to reduce liver steatosis, inflammation, and fibrosis by lowering the mRNA expression of PNPLA3 [104]. Another trial in phase II is using ASOs targeting diacylglycerol acetyltransferase 2 (DGAT2), an enzyme that catalyzes the final reaction in the synthesis of triglycerides. This antisense nucleotide works to reduce the production of DGAT2 and therefore decrease triglyceride synthesis in the liver [105,106].

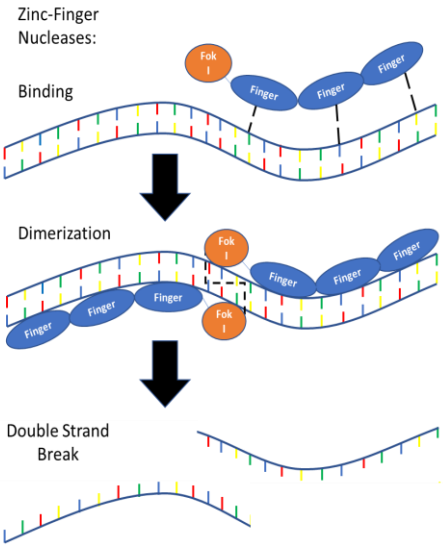

Fig. 1. The Action of Zinc-Finger Nucleases

The Zinc-Finger Nuclease (ZFN) binds to one strand of DNA using multiple base pairs, with the FokI nuclease used as a cleavage domain. Dimerization occurs when another ZFN attaches to the complementary DNA strand, creating a double stranded break

The field of genetic therapies continues to grow rapidly in all fields as it does in the sphere of hepatology. The continued success of genetic therapies in other fields of medicine will fuel the growth in hepatology. There is no definitive endpoint to genetic therapy research. There are limitations to the field of genetic therapies not only on the technical/research and development side, but also in funds of knowledge of providers prescribing these medications, and public distrust in genetic therapies. A meta-analysis reported patients were more comfortable with gene therapies if patients discussed risks and benefits with their patients [107].

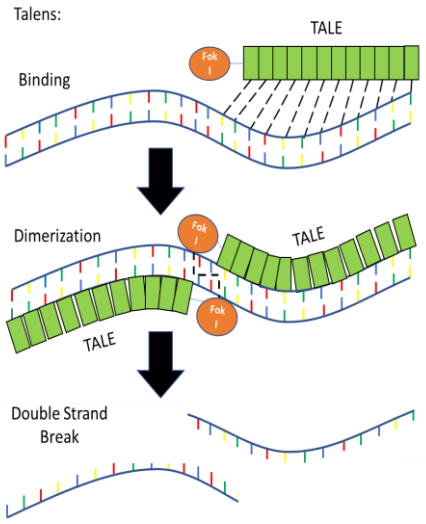

Fig. 2. Transcription activator-like effector nucleases (TALENs)

Transcription activator-like effector nucleases (TALENs) are a set of protein codes flanked by a C-terminal domain and an N-terminal domain and attached to a FokI domain. One TALEN attaches to a strand of DNA and the FokI nuclease is used as a cleavage domain. Another TALEN attaches the complementary DNA strand, dimerization occurs and creates a double stranded break.

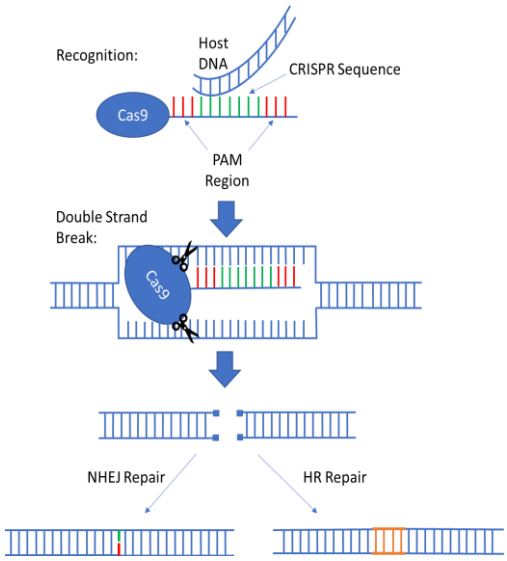

Fig. 3. cluster regulated interspaced short palindromic repeats with associated cast 9 proteins (CRISPR/cas9)

CRISPR sequences are associated protospacer adjacent motif (PAM) regions used to help break down DNA, with associated Cas9 adjacent.The CRISPR sequence is used to create protein complexes to identify specific DNA sequences. After the complimentary region of DNA is identified, the Cas9 protein creates a double-strand break. After the DNA break occurs, the strand is repaired through non-homologous end joining (NHEJ) or innate homology repair (HR)

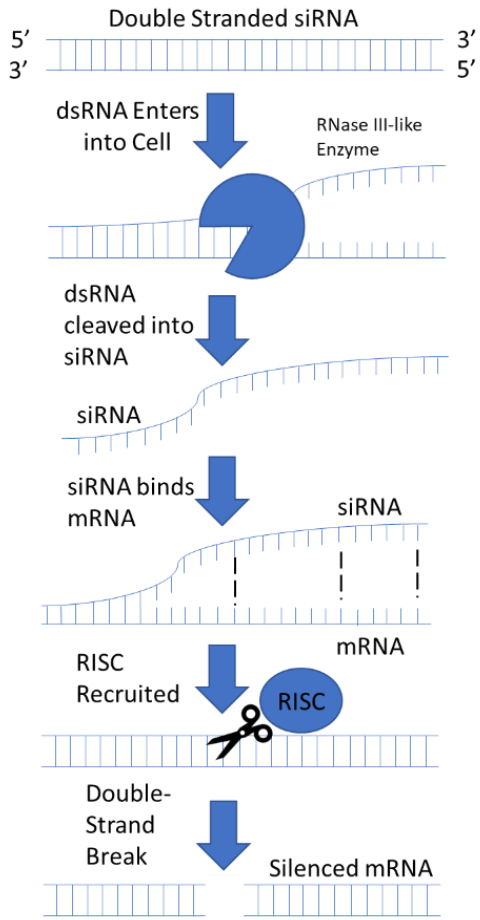

Fig. 4. Double Stranded short interfering RNA (siRNA)

Double stranded RNA enters the cell and an Rnase III-like enzyme cleaves the double stranded RNA into the single strand siRNA. siRNA then binds to mRNA an RNA-induced silencing complex is recruited once binding is accomplished. A double-stranded DNA break is created, resulting in silencing of the mRNA

Table 1. List of genetic therapy clinical trials for liver diseases

Disease	Study #	Vector	Modification type	Molecule	Target	Clinical phase	Route	Ref
A1AT liver disease	NCT03946449	cholesterol - conjugated siRNA	siRNA	Inhibits AAT mRNA	AAT mRNA	II (active)	subcutaneous	[107,108]
	NCT02363946	siRNA	Dynamic polyconjugate	Silence AAT gene expression	AAT gene	I (terminated)	IV infusion	[108]
	NCT04764448	GalNAc-siRNA	siRNA	siRNA targeting SERPINA1		II (recruiting)	subcutaneous	[109]
	NCT02503683					I (Terminated)		[110]
	NCT04174118	GalNAc-siRNA	siRNA			(I) active	subcutaneous	[111]
Acute hepatic porphyria	NCT03338816	GalNAc-siRNA conjugate	siRNA	siRNA against ALAS1	ALAS1	III (completed)	IV infusion	[75]
Acute intermittent porphyria	NCT02082860	AAV5	Gene transfer	Liver specific-promoter for Porphobilinogen deaminase expression	Porphobilinogen deaminase	I (completed 2014)	IV infusion	[39]
Acute intermittent porphyria	NCT02452372	GalNAc-siRNA conjugate	siRNA	Inhibitor of hepatic aminolevulinic acid synthase 1 (ALAS1)	Delta-aALAS1	I (completed)	Subcutaneous	[76]
Chronic HBV	NCT02826018	GalNAc-siRNA conjugate	siRNA		HBV mRNAs	I (terminated)		[112]
	NCT03772249	GalNAc-siRNA conjugate	siRNA			I (Complete)	subcutaneous	[81]
	NCT03672188	GalNAc-siRNA conjugate	siRNA		HBV transcripts			[84]
	NCT02981602		ASO		HBV messenger RNAs	II (Completed)	subcutaneous	[80]
	NCT03365947	GalNAc-siRNA conjugate	siRNA		HBV mRNAs	I/II (completed)	subcutaneous	[113]
Crigler Najjar	NCT03466463	AAV	Gene transfer	UDP glucuronosyltransferase 1 (UGT1A1) transgene	UGT1A1	N/a (recruiting)	IV infusion	[49]
	NCT03223194	AAV8	Gene transfer	UGT1A1 gene	UGT1A1	I (terminate due to sponsor decision)	IV infusion	[50]
Fabry	NCT04040049	AAV	Gene transfer	alpha galactosidase gene	alpha galactosidase	I/II (recruiting)	IV infusion	[43]
	NCT04046224	AAV 2/6	Gene transfer	cDNA of Alpha galactosidase	Alpha galactosidase	I/II (recruiting)	IV infusion	[42]
	NCT04519749	AAV	Gene transfer	Codon-optimized full length human GLA transgene driven by CAG promoter	Alpha galactosidase	I/II (recruiting)	IV infusion	[44]
Familial hypercholesterolemia	NCT00004809	Autologous hepatocytes	Gene transfer	Low-density lipoprotein (LDL) receptor gene	LDL receptor	I (completed)	Inferior mesenteric vein infusion	[114]

Disease	Study #	Vector	Modification type	Molecule	Target	Clinical phase	Route	Ref
	NCT02314442	GalNAc-siRNA conjugate	siRNA	PCSK9 inhibitor	PCSK9	I (completed)	Subcutaneous	[115]
	NCT03851705	GalNAc-siRNA conjugate	siRNA	PCSK9 inhibitor	PCSK9	III (completed)	Subcutaneous	[82]
	NCT02651675	AAV8	Gene transfer	Human low-density lipoprotein receptor gene	Low Density Lipoprotein Receptor	I/II (completed)	IV infusion	[41]
	NCT02709850			Was discontinued due to phase 2b results				[116]
Glycogen Storage Disease Type 1a (GSD1a)	NCT05095727		mRNA	mRNA encoding Glucose 6-phosphatase	Glucose 6-phosphatase	I (recruiting)	IV infusion	[117]
Hemophilia A	NCT02576795	AAV5	Gene transfer	B domain deleted Factor VIII	Factor VIII	I/II (ongoing)	IV infusion	[27]
	NCT03370913	AAV	Gene transfer	B-domain-deleted factor VIII	Factor VIII	III (active)	IV infusion	[29]
	NCT03003533	AAV	Gene transfer	B domain deleted Factor VIII	Factor VIII	I/II (recruiting)	IV infusion	[28]
	NCT03061201	AAV 2/6	Gene transfer	cDNA containing B domain deleted Factor VIII	Factor VIII	II (ongoing)	IV infusion	[31]
	NCT03588299	AAV	Gene transfer	B domain deleted Factor VIII	Factor VIII	I/II (ongoing)	IV infusion	[32]
	NCT03370172	AAV8	Gene transfer	B domain deleted Factor VIII	Factor VIII	I/II (ongoing)	IV infusion	[30]
Hemophilia A and B	NCT03549871	GalNAc-siRNA	siRNA	siRNA targeting antithrombin	Antithrombin	III (completed)	subcutaneous	[118]
	NCT03754790	GalNAc-siRNA	siRNA		antithrombin	III (active)	subcutaneous	[119]
	NCT03417245	GalNAc-siRNA	siRNA		antithrombin	III (active)	subcutaneous	[120]
	NCT03417102	GalNAc-siRNA	siRNA		antithrombin	III (active)	subcutaneous	[121]
Hemophilia B	NCT00076557	AAV	Gene transfer	Human Factor IX	Factor IX	I/II was terminated with no results	Hepatic artery injection	[25]
	NCT03569891	AAV5	Gene transfer	Padua variant of a codon optimized factor IX gene	Factor IX	III (active)	IV infusion	[16]
	NCT00979238	AAV	Gene transfer	codon-optimized factor IX transgene	Factor IX	I (ongoing)	IV infusion	[19]
	NCT02396342	AAV5	Gene transfer	Codon optimized human factor IX gene	Factor IX	I/II Completed	IV infusion	[26]
	NCT01687608	AAV8	Gene transfer	Factor IX gene	Factor IX	I/II ongoing	IV infusion	[23]
	NCT03489291	AAV5	Gene transfer	Padua variant of a codon optimized factor IX gene	Factor IX	IIb	IV infusion	[18]
	NCT03369444 NCT03641703	AAV	Gene transfer	Transgene cassette including liver-specific promoter (FRE1) and a partially codon-optimized gene encoding factor IX with	Factor IX	I/II (terminated) Long term observational study currently	IV infusion	[20,22]

Disease	Study #	Vector	Modification type	Molecule	Target	Clinical phase	Route	Ref
				a gain of function		active		
	NCT02618915	AAVRH10	Gene transfer	Factor IX	Factor IX	I/II terminated due to sponsor decision	IV infusion	[24]
	NCT02695160 NCT04628871	AAV6	Zinc Finger mediated	Factor IX	Factor IX at albumin locus	I (terminated)	IV infusion	[17,21]
Hepatocellular Carcinoma	NCT02191878	Stable nucleic acid lipid particle	siRNA	Decreased expression of polo-like kinase 1 (PLK 1) expression	PLK1	I/II (completed)	IV infusion	[122]
	NCT03780049	AAV5		Oncolytic activity		III (recruiting)	IV infusion	[123]
Hereditary Transthyretin amyloidosis with polyneuropathy	NCT04601051	Lipid nanoparticles (LNPs)	CRISPR/CAS9	Decreased production of both wild-type and mutant transthyretin (TTR) protein	TTR in hepatocytes	I (recruiting)	IV infusion	[69]
Methylmalonic acidemia	NCT03810690		mRNA			Study withdrawn before dosing		[124]
	NCT04581785	AAV	LK03 capsid	Methylmalonyl-CoA mutase gene at albumin locus		I/II (recruiting)	IV infusion	[40]
	NCT04899310 NCT05295433	Lipid nanoparticle	mRNA	Methylmalonyl-coenzyme A (CoA) mutase (MUT) gene	MUT	I/II (recruiting)	IV infusion	[125,126]
MPS I	NCT02702115 NCT04628871	AAV8	Zinc finger nuclease	Alpha-L-Iduronidase transgene	Alpha-L-iduronidase gene at the albumin locus	I/II (subjects rolled over to long-term follow up study)	IV infusion	[21,45]
MPS II	NCT03041324 NCT04628871	AAV	Zinc finger nuclease	Iduronate 2 – sulfatase gene	Iduronate 2 – sulfatase gene at the albumin locus	I/II(subjects rolled over to long-term follow up study)	IV infusion	[21,46]
MPS IV	NCT03173521	AAV8	Gene transfer	Liver specific thyroxine binding globulin promoter	Arylsulfatase B gene	I/II (active)	IV Infusion	[47]
NASH	NCT04932512	Ligand conjugated	ASO		Diacylglycerol acyltransferase 2	II (recruiting)	Subcutaneous	[104]
	NCT04483947	Ligand conjugated	ASO	Patatin-like phospholipase domain containing protein 3	mRNA expression of PNPLA3	I (recruiting)	subcutaneous	[103]
OTC deficiency	NCT03767270	Lipid based nanoparticles	mRNA	Ornithine transcarbamylase gene	OTC	I/II withdrawn (program discontinued)		[127]
	NCT00004498	AAV	Gene transfer	Ornithine transcarbamylase gene	Ornithine transcarbamylase	I (terminated)	Hepatic Artery	[35]
	NCT00004386	AAV	Gene transfer	Ornithine transcarbamylase	Ornithine	I (terminated)	Intrahepatic	[34]

Disease	Study #	Vector	Modification type	Molecule	Target	Clinical phase	Route	Ref
				gene	transcarbamylase		infusion	
	NCT02991144	AAV8	Gene transfer	Ornithine Transcarbamylase gene	Ornithine transcarbamylase	I/II (completed)	IV infusion	[36]
	NCT05345171	AAV8	Gene transfer	Ornithine Transcarbamylase gene	Ornithine transcarbamylase	III (recruiting)	IV infusion	[33]
Phenylketonuria	NCT03952156	AAVHSC15	Gene transfer	Functional copy of phenylalanine hydroxylase gene	Phenylalanine hydroxylase	I/II (recruiting)	IV infusion	[38]
	NCT04480567	AAV5	Gene transfer	Phenylalanine hydroxylase gene	Phenylalanine hydroxylase	I/II (active)	IV infusion	[37]
Primary hyperoxaluria	NCT05001269	GalNAc-siRNA conjugate	siRNA	siRNA against hepatic LDH	Hepatic lactate dehydrogenase (LDH)	II (recruiting)	subcutaneous	[128]
	NCT03681184	GalNAc-siRNA	siRNA	Decreased hepatic oxalate production	Glycolate oxidase	III (active) approved for sale	Subcutaneous injection	[77]
Propionic acidemia	NCT04159103		mRNA			I/II (recruiting)	IV infusion	[129]
Transthyretin TTR amyloidosis	NCT03728634	GalNAc-ASO conjugate	Antisense oligonucleotide	ASO against TTR mRNA	TTR mRNA	I/II (completed)	IV infusion	[130]
	NCT05071300	Ligand-conjugated antisense	Antisense oligonucleotide	ASO against TTR	TTR protein	III (recruiting)	Subcutaneou	[89]
	NCT03759379	GalNAc-siRNA conjugate	siRNA	siRNA against transthyretin protein	Transthyretin protein	III (active)	subcutaneous	[131]
	NCT04153149			May not be relevant as this is cardiac amyloidosis				[132]
Wilson’s disease	NCT04884815	AAV9	Gene transfer	ATP7B Gene	Copper transporting ATPase 2	I/II (recruiting)	IV infusion	[48]

Table 2. List of animal studies using genetic therapy for liver diseases

Disease	Target Organ	Vector	Modification type	Molecule	Target	Route	Ref
AATD	Mouse liver	AAV	CRISPR/Cas9	Guide RNA	hSERPINA1	Hydrodynamic tail vein injection	[133]
HBV	Mouse liver	TALEN expressing plasmid	TALEN	HBV target DNA and pairs of left and right TALEN expressing plasmids	Sites within the S/pol, C/pol, and pol ORFs of HBV genome	Hydrodynamic injection	[64]
Hemophilia A and B	Mouse liver	AAV8	ZFN	Human F9 Gene	Intron 1 of Human F9 Gene	IV infusion	[134]
	Mouse liver	AAV8	ZFN		Albumin locus	IV infusion	[135]
	Mouse liver	AAV8	ZFN		Human IDS at the albumin locus		[136]
Hemophilia B		AAV9	CRISPR/Cas9	Guide RNA	Murine Factor IX gene	Hydrodynamic tail vein injection	[137]
HTI	Mouse liver	pX330 plasmid	CRISPR/Cas9	pX330 plasmids expressing Cas9 and a single guide RNA	Fumarylacetoacetate hydrolase (fah) locus	Hydrodynamic tail vein injection	[138]
	Mouse liver	Lipid nanoparticle and AAV	CRISPR/Cas9	U6-sgRNA, homology directed repair template	Exon 8 of Fumarylacetoacetate hydrolase (fah)	IV injection	[139]
	Mouse liver	pX330	CRISPR/Cas9	Guide RNA sequences	Introns adjacent to exons 3 and 4 of hydroxyphenylpyruvate dioxigenase gene	Hydrodynamic tail vein injection	[140]
	Mouse liver	Plasmid DNA	CRISPR/Cas9	Adenine base ditor and a single guided RNA	Exon 8 of Fumarylacetoacetate hydrolase (fah)	Hydrodynamic tail vein injection	[141]
MPS I	Mouse liver	AAV8	CRISPR/Cas9	Albumin locus of hepatocytes	Alpha-L-iduronidase		[91]
MPS II	Mouse liver	AAV2/AAAV8	ZFN	Human IDS coding sequence	Intron 1 of the albumin locus	IV infusion?	[61]
Transthyretin amyloidosis	Mouse liver	Lipid nanoparticles	CRISPR/Cas9	Cas9 mRNA with single guide RNA	Transthyretin gene	Hydrodynamic tail vein injection	[142]

Abbreviations: *ATD, alpha-1 antitrypsin deficiency; AAV, Adeno-associated vector; ABE, adenine base editing; Ad, adenovirus; ALS, amyotrophic lateral sclerosis; CAR, chimeric antigen receptor; CRISPR/Cas, clustered regularly interspaced short palindrome repeats-associated Cas nuclease; dCas9, dead Cas9; DMD, Duchenne muscular dystrophy; gRNA, guide RNA; HBV, hepatitis B virus; HDR, homology-directed repair; HIV, human immunodeficiency virus; HITI, homology-independent targeted integration; HR, homologous recombination; HTI, hereditary tyrosinemia; LCA, Leber's congenital amaurosis; LNP, lipid nanoparticles; NHEJ, nonhomologous end-joining; PNA, peptide nucleic acids; RNP, ribonucleoprotein; SCD, sickle cell disease; sgRNA, single-guide RNA; TALEN, transcription activator-like effector nuclease; ZFN, zinc-finger nuclease.*

4. CONCLUSION

The future implications for genetic therapies have no bounds. A majority of trials outlined in this article are either animal studies or phase 1 and phase 2 trials. Most modalities have passed animal studies showing safety and efficacy. These data points will encourage future research into different liver diseases. SiRNA seems to be the forefront of therapy modalities in trials currently. There are multiple animal trials associated with the CRISPR/Cas9 system, but very few clinical trials at this time [143]. Even with all this promising data, and studies to give validity to these concepts, there has been a lot of public mistrust over the recent years with genetic therapies, especially mRNA therapies with the advent of the COVID-19 vaccines. Future problems for gene therapy include the management of off-target effects and specifying delivery particles to decrease this effect. There still are leaps-and-bounds to be made on the efficacy and penetrance of genetic therapies and normalizing these types of therapies to the public. Gene therapy is most likely the future of medicine across the board. These concepts can be difficult to comprehend not only for patients but for clinicians that are not specialized in genetics/biochemistry. There is a need to understand these basic concepts of genetic therapy so clinicians can perform their due diligence to explain how medications work, and to give hope for patients that are struggling with liver diseases that do not have effective treatment modalities today.

CONSENT AND ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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