nature reviews gastroenterology & hepatology

https://doi.org/10.1038/s41575-022-00729-0

Review article

Check for updates

Gene therapy for liver diseases — progress and challenges

Nerea Zabaleta [1], Carmen Unzu [2], Nicholas D. Weber [3] & Gloria Gonzalez-Aseguinolaza [2,3]

Abstract

Gene therapy is poised to revolutionize modern medicine, with seemingly unlimited potential for treating and curing genetic disorders. For otherwise incurable indications, including most inherited metabolic liver disorders, gene therapy provides a realistic therapeutic option. In this Review, we discuss gene supplementation and gene editing involving the use of recombinant adeno-associated virus (rAAV) vectors for the treatment of inherited liver diseases, including updates on several ongoing clinical trials that are producing promising results. Clinical testing has been essential in highlighting many key translational challenges associated with this transformative therapy. In particular, the interaction of a patient's immune system with the vector raises issues of safety and the duration of treatment efficacy. Furthermore, several serious adverse events after the administration of high doses of rAAVs suggest greater involvement of innate immune responses and pre-existing hepatic conditions than initially anticipated. Finally, permanent modification of the host genome associated with rAAV genome integration and gene editing raises concerns about the risk of oncogenicity that require careful evaluation. We summarize the main progress, challenges and pathways forward for gene therapy for liver diseases.

Sections

[1]Grousbeck Gene Therapy Center, Schepens Eye Research Institute, Mass Eye and Ear, Boston, MA, USA. [2]Gene Therapy and Regulation of Gene Expression program, CIMA, Universidad de Navarra, IdisNA, Pamplona, Spain. [3]Vivet Therapeutics S.L., Pamplona, Spain. e-mail: ggasegui@unav.es

Key points

- Gene therapy mediated by recombinant adeno-associated virus (rAAV) vectors has emerged as a therapeutic option, with inherited liver disorders being prime targets for this strategy.
- The first gene therapy for haemophilia B has been approved by the FDA, another for haemophilia A is nearing approval, and trials of several other rAAV-based liver-targeted gene therapies have produced promising results.
- High rAAV doses (>1×10^{14} vg/kg) seem to be associated with severe adverse effects, including hepatotoxicity and immune response-associated sequelae.
- Due to the primarily non-integrative nature of rAAV genomes, loss of vector genomes during cell turnover is of essential concern for the durability of therapeutic effect, particularly in paediatric patients.
- Gene-editing strategies offer the most powerful tools for permanently correcting genetic disorders via direct modification of the genome but pose additional safety risks, such as oncogenicity, owing to insertional mutagenesis.

Introduction

The liver is a multifunctional organ responsible for many essential functions, including xenobiotic detoxification, bile production, vitamin and glucose storage, iron metabolism, regulation of hormones, production of most plasma proteins, and metabolism of carbohydrates, fat and proteins[1]. It is, therefore, the primary site of many genetic metabolic disorders, such as Gaucher disease, Fabry disease, mucopolysaccharidosis type I (MPS I), MPS II, MPS IV and Pompe disease, and has a central role in genetic diseases associated with deficiencies of secreted proteins, such as haemophilia[2–10]. Consequently, the liver is a prime target for gene therapy, and many liver-targeted gene therapy approaches are under investigation or in clinical development[2–10].

Gene therapy involves the use of genetic material for the treatment or prevention of diseases. In 1972, Friedmann and Roblin first suggested the use of genetically modified viruses to treat genetic disorders[11], and the concept of gene therapy was proposed in the late 1970s following the development of recombinant DNA technology[12–15]. The first gene therapy trial in humans was performed in 1990 — a 4-year-old girl with severe immunodeficiency due to adenosine deaminase deficiency was successfully treated with T cells transduced with a retroviral vector carrying the correct version of the *ADA* gene[16]. Gene therapy has since been tested in clinical trials for a multitude of inherited diseases, cancers, and infectious diseases[17] and is progressively altering the outlook of modern medicine — six gene therapy drugs have been approved by the FDA or the EMA, and >3,000 clinical trials of gene therapy have been registered[18].

Initially, gene therapy consisted solely of gene supplementation to treat loss-of-function diseases. However, gene therapy has developed to include other approaches such as silencing of pathogenic genes[19] and precise modification of the cellular genome[20]. The development of CRISPR–Cas9 technology — the Swiss Army knife of gene editing — is one of the most exciting scientific breakthroughs of the past decade. Subsequent iterations and advancements of this technology promise to improve its precision and versatility via base editors and prime editors, among others[18,21–23]. Fundamental to the development of gene therapy has been progress in our understanding of the underlying genetic causes of diseases, improvement of gene delivery systems, and development of novel nucleic acid-based therapeutics and genome-engineering technologies (Box 1).

Despite promising results with gene therapy to date, myriad challenges remain in translating gene therapy research into the clinic, particularly safety risks. Novel approaches that involve the delivery of genetic material to cells or manipulation of genomic DNA require great caution and careful evaluation of all possible outcomes before their approval. Indeed, despite years of drug development, the first liver-targeted gene therapy is still only nearing regulatory approval — priority review of the Biologics License Application has been accepted by the FDA for etranacogene dezaparvovec for the treatment of haemophilia B[24] — and, in June 2022, the EMA recommended granting a conditional marketing authorization in the EU for valoctocogene roxaparvovec, the first gene therapy for the treatment of severe haemophilia A[25].

In this Review, we discuss the most recent progress in the development of gene therapy for inherited liver disorders, the challenges associated with this technology, and potential solutions currently being considered and tested.

Fundamentals of rAAV vectors

Wild-type adeno-associated viruses (AAVs) are small, non-pathogenic viruses composed of an icosahedral protein capsid with a diameter of 20–25 nm, and a single-stranded DNA genome of ~4.7 kb flanked by two inverted terminal repeats (ITRs)[26,27]. AAVs can infect multiple vertebrate species, including humans and non-human primates[28]. AAVs are naturally replication deficient and rely on co-infection with a helper virus to replicate, a distinctive feature that is highly advantageous for their use as delivery vectors. Owing to these characteristics and the relatively low immunogenicity of AAVs compared with other viral vectors[29], recombinant AAVs (rAAVs) have become the most commonly used vectors for gene delivery[30,31].

rAAVs share the same capsid sequence and DNA structure, including ITRs, with wild-type AAVs, but all coding sequences have been replaced by a transgene expression cassette[32]. They are produced through plasmid transfection of mammalian cell lines (HEK293 or HeLa cells) or recombinant baculovirus infection of insect cells.

Box 1

Key factors in the progress of gene therapy

- Improved understanding of the underlying genetic causes of inherited disorders as a result of advances in sequencing technologies and bioinformatics tools.
- Improved understanding of the pharmacokinetics, stability and toxicity of gene delivery vectors.
- Development of novel and more efficient genetic-based in vivo and in vitro therapeutic models.
- Development of sophisticated methods for gene delivery to target tissues.
- Large-scale viral vector manufacturing capabilities.

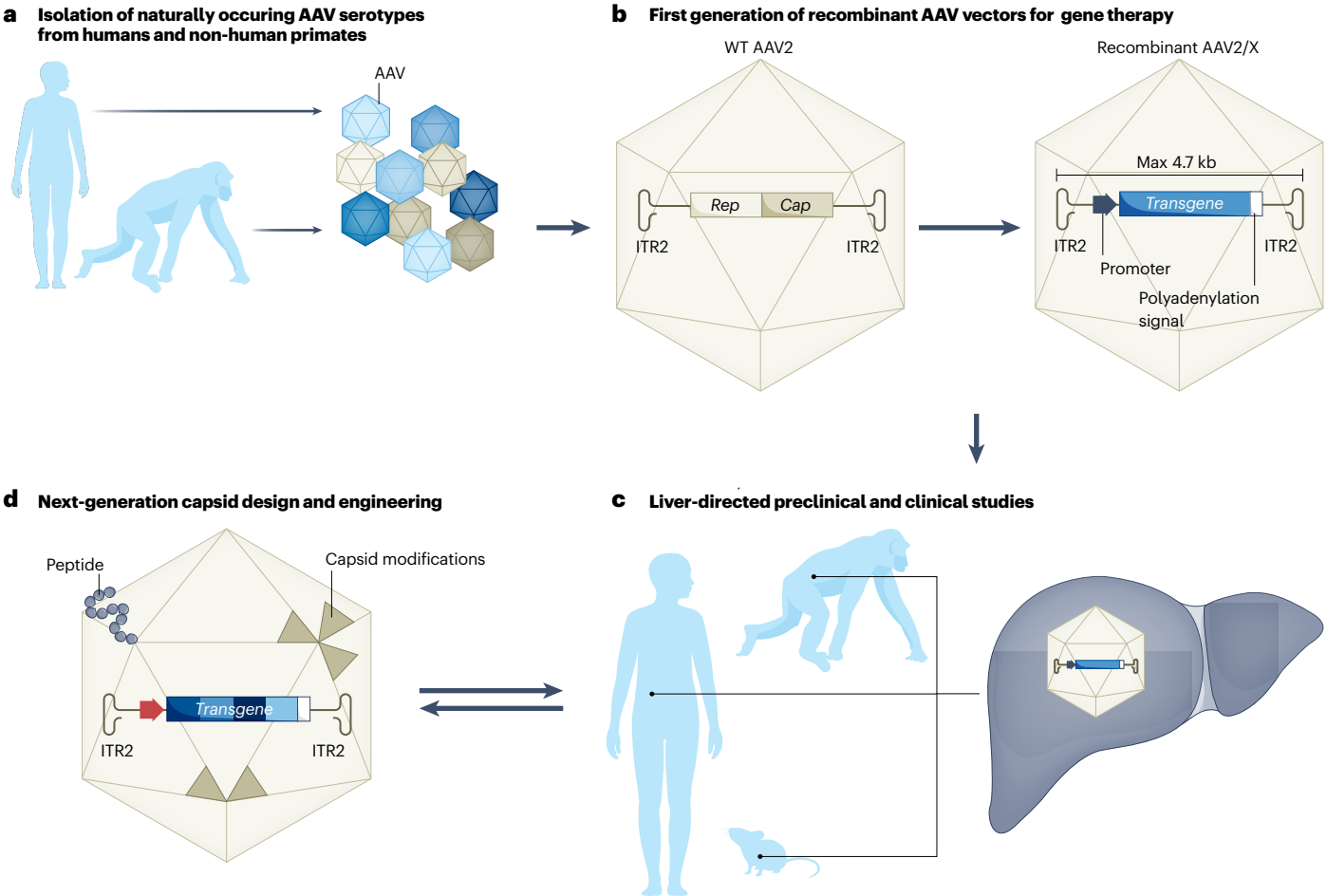

Fig. 1 | The process of developing recombinant AAV gene delivery vectors. **a**, Naturally occurring wild-type (WT) adeno-associated virus (AAV) serotypes are isolated from various tissues of humans and non-human primates[20]. **b**, Recombinant AAVs are created by replacing viral genes with recombinant DNA sequences that contain the therapeutic expression cassette. The inserted sequences are flanked by the AAV serotype 2 inverted terminal repeats (ITR2). These AAV2-based genomes can be packaged into different capsid serotypes (AAV2/X). **c**, Recombinant AAV vectors are evaluated in preclinical and clinical studies to study their safety and therapeutic effects in inherited liver disorders. **d**, Data on transduction, expression, biodistribution and toxicity from the preclinical and clinical studies are used to develop next-generation recombinant AAVs to improve specific therapeutic characteristics. Strategies include capsid engineering (for example, rational and in silico design, capsid protein shuffling, peptide insertion and mutagenesis, and natural variant discovery) to reduce immunogenicity and off-target transduction, and sequence modifications (for example, optimizing transgene and regulatory sequences and minimizing cytosine–guanine dinucleotide motifs) to improve transgene expression and tissue tropism and reduce immunostimulatory side effects.

This transfection or infection introduces into the cells the essential AAV genes (*rep* and *cap*) and helper virus genes that are necessary for viral production, together with the recombinant genomic construct carrying the therapeutic expression cassette flanked by the ITRs[32]. This recombinant genome is assembled inside the viral capsid within the cells to produce the final rAAV.

rAAV-mediated delivery of genetic material to cells in vivo starts with the interaction of the capsid with surface glycans and receptors, followed by endocytosis, intracellular endosomal trafficking and escape from the late endosome. Entry into the nucleus occurs via nuclear pores owing to a nuclear localization signal sequence present in the capsid proteins. Once in the nucleus, the AAV genome is released from the capsid and forms episomes, most of which are concatemers created by intermolecular interactions between ITRs[32–35]. Currently, rAAVs are the most efficient vector for the delivery of genetic material to the hepatocyte nucleus that leads to long-term expression of a therapeutic transgene[30].

Most AAV capsids currently used for liver gene therapy are based on naturally occurring serotypes isolated from human and non-human primates[31,36] (Fig. 1), though AAVs have also been isolated from cows, horses, mice, goats and birds[37]. More advanced bioengineered viral vector capsids with more desirable features, such as lower immunogenicity, modified organ or cell tropism, and higher production yields, are being developed (Fig. 1), some of which are being used in ongoing clinical trials[31,36].

The liver as a gene therapy target

Important in the use of rAAV vectors for liver-targeted gene therapy is the tolerogenic nature of the liver[38–40], demonstrated by the fact that immunosuppression can be withdrawn in ~20% of patients who

receive a liver transplant[41]. Hepatic immune tolerance is exploited by several hepatotropic pathogens, including hepatitis B and C viruses, *Plasmodium* species, adenoviruses, and several non-pathogenic viruses such as AAVs[39,42,43]. The immunotolerogenic properties of the liver prevent the development of immune responses against AAV-delivered transgenes, thereby facilitating long-term expression[39]. Furthermore, the use of hepatocyte-specific promoters and regulatory elements that restrict transgene expression to these cells induces tolerance to the recombinant protein product[44,45].

Almost all naturally occurring AAV serotypes transduce the liver efficiently upon systemic administration[30,31]. This efficiency is due to the unique dual blood supply to the liver, which receives approximately 1 l of blood per minute, and the fenestrations of liver endothelial sinusoidal cells with diameters of 50–300 µm, which allow AAV particles in the blood to directly access hepatocytes[46]. Consequently, rAAVs provide a reliable vector for targeting inherited liver disorders. This high transduction efficiency of rAAVs combined with the tolerogenic nature of the liver and the high abundance of inherited liver disorders makes the liver an ideal target for rAAV-based gene therapies.

Liver-targeted gene therapies

In preclinical studies, rAAV-mediated gene augmentation in the liver has had therapeutic efficacy in several indications, including haemophilia, glycogen storage diseases and various metabolic diseases[2–10]. In particular, the most extensive experience has been gained in the treatment of hereditary haemophilias (Table 1). These genetic conditions do not involve liver pathology, but the liver is the source of the disease owing to an inability to produce and secrete the coagulation factors necessary for blood clotting. The full effects and lessons learned from the preclinical and clinical studies of this approach are discussed in more detail below, but phase I–II clinical trials have demonstrated long-term transgene expression with good safety profiles (Table 1). In November 2022, the FDA approved the Biologics License Application for one rAAV product to treat haemophilia B, etranacogene dezaparvovec, after successful completion of the phase III clinical trial[47–49]. Another treatment for haemophilia B (fidanacogene elaparvovec[50,51]) and two for haemophilia A (giroctocogene fitelparvovec[52,53] and valoctocogene roxaparvovec[54–58]) are currently in phase III clinical trials (Table 1).

In addition to haemophilia, the therapeutic efficacy of rAAV-mediated gene augmentation has been demonstrated in preclinical studies of several genetic metabolic liver indications, including familial hypercholesterolaemia[59], Crigler–Najjar syndrome[8], ornithine transcarbamylase (OTC) deficiency[2], phenylketonuria[4], Wilson disease[60], acute intermittent porphyria (AIP)[3], progressive familial intrahepatic cholestasis[5], glycogen storage disease type Ia (GSD1a)[61] and methylmalonic acidaemia (MMA)[62]. Consequently, the number of clinical trials for genetic liver disorders is continually increasing, and many have produced promising outcomes in patients (Table 1).

For example, six of nine patients with OTC deficiency responded to treatment with rAAV-*OTC*, and three were considered to be complete responders[63,64]. In patients with phenylketonuria, an inherited disorder in which mutations in the gene that encodes phenylalanine hydroxylase (PAH) lead to the accumulation of toxic levels of phenylalanine in the blood, gene augmentation therapy with an rAAV carrying the *PAH* gene led to meaningful reductions in phenylalanine levels and increases in tyrosine levels[65,66]. In a phase I–II clinical trial in nine patients with GSD1a, treatment with an rAAV-expressing glucose-6-phosphatase (G6Pase) significantly reduced the need for cornstarch (the standard of care for GSD1a) and improved glucose control and other metabolic parameters in all nine individuals[67,68]. Exciting results are also emerging from trials of rAAV-mediated delivery of the enzyme uridine diphosphate glucuronosyltransferase 1A1 (UGT1A1) in patients with Crigler–Najjar syndrome. The highest doses significantly reduced bilirubin levels and, most importantly, enabled two of three patients to withdraw from phototherapy (the third was waiting until sufficient time had passed after treatment to undergo analysis for phototherapy withdrawal)[69,70]. On the basis of the information presented by the sponsors and positive outcomes in phase I–II clinical trials, phase III studies for patients with OTC deficiency[71] (NCT05345171) and GSD1a[72] (NCT05139316) were initiated in October 2022.

Limitations of liver-targeted gene therapy

Despite the promising outcomes in clinical trials of rAAV-based gene therapy, several considerable limitations remain. In particular, host immune responses can be triggered by the capsid, foreign DNA elements contained in the vector genome and the protein product of the transgene, thereby preventing effective delivery and long-term expression of the transgene[73]. Immune system activation can also reduce the efficacy of the gene therapy product or cause adverse events. There are myriad additional areas of concern that merit close attention, including hepatotoxicity, complement activation, neurotoxicity, genotoxicity and loss of effect, as discussed in the sections below.

Adaptive immune responses

Antibody response to rAAVs. Neutralizing antibodies are the first defence barrier mounted by the host against wild-type AAVs (Fig. 2). In general, humans are exposed to natural AAV infection early in life[74,75] so the seroprevalence of antibodies against naturally occurring AAV serotypes is universally high – typically 30–80%[74–76]. Strong similarities between AAV serotypes can lead to a broad, cross-neutralizing antibody response[77]. Currently, screening for neutralizing antibodies to select people who are AAV seronegative is a requirement for inclusion in most clinical studies of AAV gene therapy, as even low antibody titres can prevent successful liver transduction when the AAV is administered systemically[78,79]. Furthermore, gene therapy strategies that require re-administration will inevitably be hindered by neutralizing antibodies that are generated upon the initial treatment.

One notable exception to the requirement for exclusion of people with pre-existing neutralizing antibodies is in trials that involve the AAV5 serotype. For example, in the HOPE-B phase III trial, patients with haemophilia B who were seropositive for AAV5 antibodies received a single dose of an rAAV5 vector that encodes coagulation factor IX at a dose of 2×10^{13} viral genomes per kilogram (vg/kg), and the findings suggested that humoral immunity did not prevent efficient gene delivery, as factor IX expression was detected in all patients[47]. However, whether this effect is related to the dose or the rAAV5 serotype is yet to be elucidated. In addition, the baculovirus production system used to produce the rAAV might have affected vector immunogenicity and susceptibility to neutralization[47]. Preclinical dose-escalation studies in animals with different neutralizing antibody titres would be required to confirm these findings and their implications for rAAV-mediated gene therapy.

Several strategies are under investigation to overcome neutralizing antibodies as a barrier to successful AAV transduction, including plasmapheresis and immunoadsorption[80,81], use of empty capsids as decoys[82], column-based capsid-specific antibody removal[83], administration of IgG-cleaving endopeptidases (such as IdeS or IdeZ[84–87]), and capsid engineering to modify or replace the epitopes targeted

Table 1 | Liver-directed gene therapy clinical trials

Indication	Therapeutic agent	Trial phase (status)	Available outcomes	Refs.
Haemophilia B	ssAAV2-F9WT	Phase I–II (terminated)	Successful liver transduction; elevation of transaminases, leading to loss of expression in patients who received high doses; rAAV neutralization by antibody titre above 1:5	92,224
	scAAV8-F9WT	Phase I (not recruiting, active)	Long-term therapeutic factor IX expression associated with clinical improvement; alanine aminotransferase rise, controlled after prednisolone treatment; no late toxic effects reported	93,225,226
	ssAAV5-coF9WT	Phase I–II (completed)	With low dose, annualized factor IX use reduced by 81%, mean ASBR reduced by 53%; with high dose, annualized factor IX use reduced by 73%, mean ASBR reduced by 70%	227,228
	ssAAV.SPARK100-F9Padua (SPK-9001)	Phase I–II (completed)	Sustained therapeutic expression of factor IX coagulant activity after gene transfer in 10 participants; termination of baseline prophylaxis and the near elimination of bleeding and factor use; no serious adverse events during or after vector infusion	94
		Phase III (active)	Ongoing	50
	scAAV8-F9Padua (BAX 335)	Phase I–II (active)	Sustained therapeutic factor IX activity of ~20%, without bleeding or replacement therapy, for 4 years in 1 patient; corticosteroid treatment did not stabilize factor IX activity loss; 4 serious adverse events in 3 participants, all considered unrelated to BAX 335; no deaths	95,229
	ssAAV5-coF9Padua (AMT-061)	Phase IIb (active)	Mean factor IX activity of 31% at week 6, increasing to 47% at 26 weeks; sustained activity of >40% in 2 participants; associated with complete bleed cessation with no need for factor IX replacement therapy up to 26 weeks	47,230
	ssAAVS3-coF9Padua (FLT180a)	Phase I–II (terminated)	Factor IX activity levels ≥50% in 7 of 8 patients treated with the three highest doses; normal levels of factor IX activity achieved with relatively low vector doses; loss of transgene expression early owing to transaminitis in 1 patient	231,232
	ssAAVrh10-coF9WT (DTX-101)	Phase I–II (terminated)	Improved levels of factor IX in all patients during post-treatment follow-up; increased ALT levels in 5 of 6 patients; trial was discontinued	233,234
Haemophilia A	ssAAV5-coBDDF8 (BMN-270)	Phase I–II (active)	Transgene expression and haemostatic response for up to 5 years; most common adverse events associated with the treatment were transient, asymptomatic mild-to-moderate ALT elevations	55,235
	ssAAVLK03-coBDDF8 (SPK-8011)	Phase I–II (recruiting, active)	Sustained factor VIII expression in 16 of 18 participants who received SPK-8011 permitted discontinuation of prophylaxis and a reduction in bleeding episodes; some participants received glucocorticoids within 52 weeks after vector administration to prevent or treat a presumed AAV capsid immune response; 17 vector-related adverse events, including 1 serious, and 16 glucocorticoid-related adverse events	165,236
	ssAAV6-coBDDF8 (PF-07055480)	Phase I–II	Increases in factor VIII levels in the mild-to-normal range, with sustained bleeding control; generally well tolerated; most commonly reported treatment-related adverse events included elevated liver enzymes and infusion-related reactions; treatment-related serious adverse events reported in 1 patient	237
		Phase III (recruiting, active)	Ongoing	53
	ssAAVhu37-coBDDF8 (DTX-201)	Phase I–II (active)	Sustained factor VIII levels (≥5%) for up to 16 months in 5 of 6 patients; all patients in two cohorts off prophylaxis since ~6 weeks after gene transfer; no serious adverse events were reported before 2020	238,239
	ssAAV8-coBDDF8 (TAK-754)	Phase I–II (active)	Factor VIII activity peaked 4–9 weeks after infusion but declined during tapering of corticosteroids; factor VIII prophylaxis resumed in 2 of 4 patients; minor transaminase elevation	240
Ornithine transcarbamylase deficiency	scAAV8-OTC (DTX301)	Phase I–II (completed)	Ammonia control maintained or improved in all 9 treated patients; 3 patients who received the highest dose were considered complete responders and have discontinued alternative medications and protein-restricted diets without loss of ammonia control; elevations of transaminases were controlled with steroid-reactive treatment	63,241

Table 1 (Continued) | Liver-directed gene therapy clinical trials

Indication	Therapeutic agent	Trial phase (status)	Available outcomes	Refs.
Phenylketonuria	ssAAVHSC15-PAH (HMI-102)	Phase I–II (recruiting)	Two dose levels were generally well tolerated and led to clinically meaningful reductions in phenylalanine levels, increases in tyrosine and reductions in the phenylalanine to tyrosine ratio	65,242
	ssAAV5-PAH (BMN 307)	Phase I–II (on hold)	Trial placed on clinical hold owing to potential drug genotoxicity in a mouse preclinical study; the clinical relevance remains under investigation	243,244
	ssAAVHSC15-PAH homology arms (HMI-103)	Phase I (recruiting)	No data available	245
Acute intermittent porphyria	ssAAV5-coPBGD	Phase I (completed)	Partial symptomatic relief and a good safety profile but no reduction in porphyrin precursor levels	157,246
Methylmalonic acidaemia	ssAAVLK03-MMA integrative (hLB-001)	Phase I–II (recruiting, active)	No drug-related serious adverse events were reported in the first 2 patients but thrombotic microangiopathy developed in the subsequent, younger 2 patients	123,247
Familial hypercholesterolaemia	ssAAV8-hLDLR (RGX-501)	Phase I–II (completed)	No data available	248
Glycogen storage disease type 1a	ssAAV8-G6PC (DTX-401)	Phase I–II (completed)	Significant reductions in the need for cornstarch and improvements in glucose control and other metabolic parameters compared to baseline in all 9 patients	68,249
Wilson disease	ssAAV3B-ATP7B (VTX-801)	Phase I–II (recruiting)	No data available	250
	ssAAV9-ATP7B (UX701)	Phase I–II (recruiting)	No data available	251
Crigler–Najjar syndrome	ssAAV8-UGT1A1 (GNT0003)	Phase I–II (recruiting)	Temporary therapeutic effect in patients who received the lowest dose; significant reduction in bilirubin levels in patients treated with a higher dose; 2 patients stopped phototherapy, a third is under evaluation; good safety and tolerability	69,70
Hereditary transthyretin amyloidosis	LNP-CRISPR–Cas9 targeting *TTR* gene	Phase I (recruiting, active)	Decreases in serum TTR protein concentrations in the 6 patients treated; 96% reduction in TTR in those who received higher doses; only mild adverse events	134,252

AAV, adeno-associated virus; ALT, alanine transaminase; ASBR, annualized spontaneous bleeding rate; LNP, lipid nanoparticle; rAAV, recombinant AAV; sc, self-complementary; ss, single-stranded; TTR, transthyretin.

by neutralizing antibodies[88]. In addition, methods are being developed to prevent the generation of neutralizing antibodies upon rAAV administration to enable further doses of the vector; these approaches include immunosuppressive regimens such as nanoparticles that contain rapamycin[89].

Cellular responses to rAAVs. Upon systemic administration of rAAV, transduced liver cells and antigen-presenting cells can present capsid-derived antigens via major histocompatibility complex (MHC) class I molecules to cytotoxic $CD8^+$ T cells, which will then eliminate transduced cells from the liver[90,91] (Fig. 2). This mechanism was identified because it led to loss of factor IX expression in one participant in the first clinical trial of rAAV-mediated liver-directed gene therapy for haemophilia B[92]. Loss of factor IX expression overlapped with an asymptomatic increase in liver transaminases ~4 weeks after systemic vector administration[92]. In the second trial of a liver-targeted rAAV for the same indication, similar transient increases in transaminases occurred in the two patients who received the high dose (2×10^{12} vg/kg)[93]. However, in this case, a short course of prednisolone treatment controlled the cytotoxic $CD8^+$ T cell response, enabling long-term factor IX expression[93]. The increase in transaminases was not observed among participants who received lower doses of the vector, suggesting that the immune response was dose dependent.

Owing to this valuable experience, many clinical protocols for testing investigational rAAV drugs now include a corticosteroid regimen at the time of rAAV administration or upon an increase in transaminase levels[51,54,94–96]. The role of $CD4^+$ and $CD8^+$ T cell responses in rAAV immunogenicity has been further highlighted in a proof-of-concept trial of AAV8, in which empty capsids triggered an IFNγ response, mainly via $CD4^+$ T cell activation[97]. In this study, co-administration of the vector with nanoparticles containing rapamycin delayed the rAAV-specific T cell response[97].

Innate immune responses

Capsid proteins and vector genomes can trigger an immediate innate immune response via Toll-like receptor (TLR) pathways in liver non-parenchymal cells during internalization[98,99] (Fig. 2). Several studies have shown that innate immune responses are associated with activation of $CD8^+$ T cells[99,100].

One trigger of this innate immune response seems to be related to the mild promoter activity of ITRs. These regions of DNA have a fundamental role in AAV biology as they contain the origin of replication and the packaging signal, and they confer the ability of AAV to form episomes in the nucleus[101]. The ITRs used in rAAVs originate from wild-type AAV serotype 2 and contain a promoter sequence that drives transgene expression[101,102]. This promoter activity can produce double-stranded RNA from the genomes, which can accumulate in transduced human hepatocytes and stimulate melanoma differentiation-associated protein 5 (MDA5) sensors that trigger type I interferon expression[103].

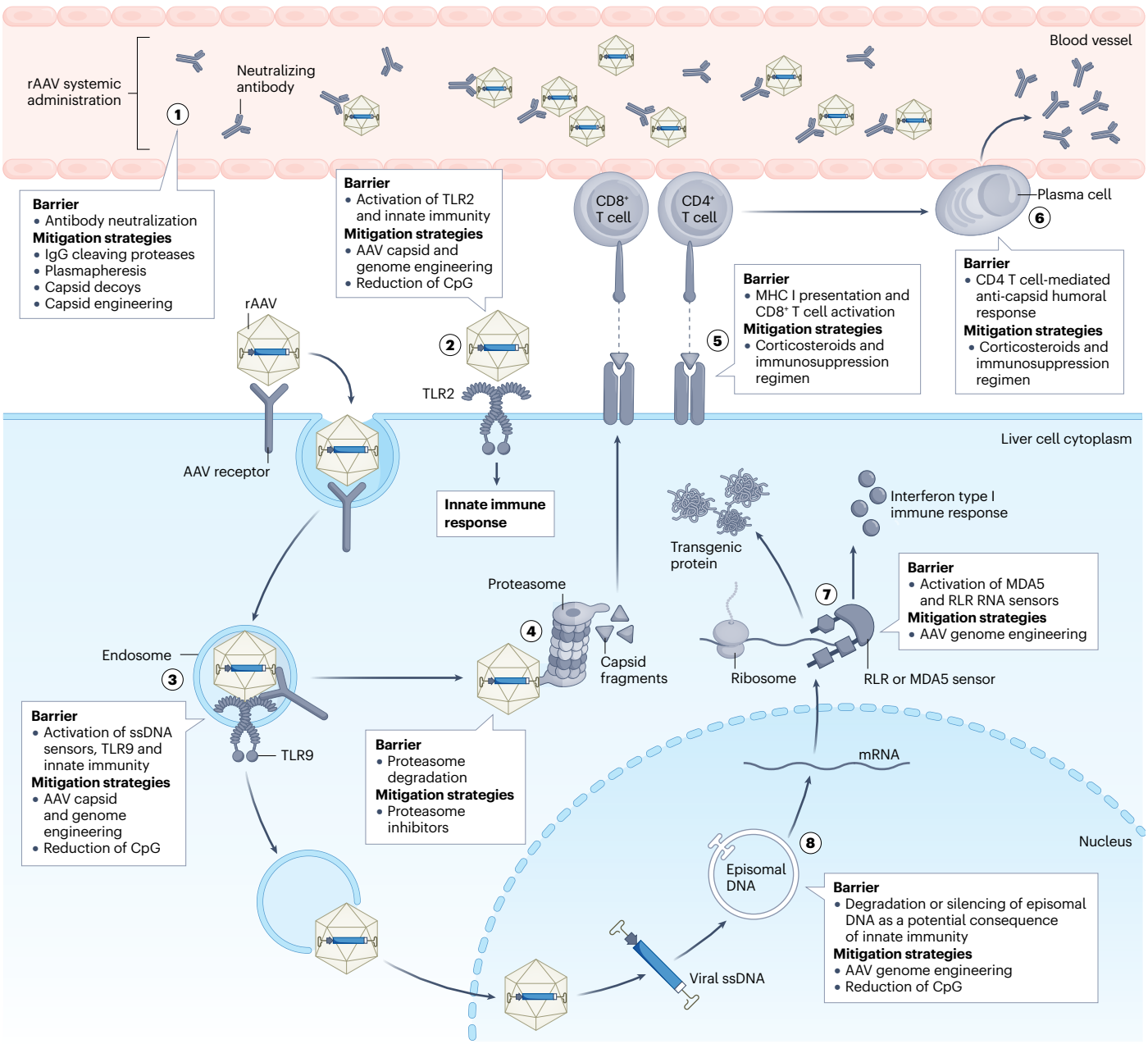

Fig. 2 | Immunological barriers to recombinant AAV-mediated gene therapy and current mitigation strategies. Pre-existing neutralizing antibodies to adeno-associated viruses (AAVs) can bind to recombinant AAV (rAAV) capsids and hamper successful liver transduction (1). Current mitigation strategies include the use of IgG-cleaving proteases, plasmapheresis, capsid decoys, capsid engineering and immunotolerance-inducing drugs. Toll-like receptors (TLRs) can recognize viral capsids and viral genome sequences as danger signals and trigger innate immune pathways (2). AAV capsid engineering and sequence modification, such as reducing cytosine–guanine dinucleotide (CpG) motifs, can mitigate innate immune responses. TLR activation in the endosome (3) can lead to AAV capsid degradation by the proteasome (4). Proteasome inhibitors, such as bortezomib, inhibit this degradation. Degradation of the AAV leads to presentation of antigenic capsid peptides by transduced liver cells via major histocompatibility complex (MHC) class I and class II proteins to $CD8^+$ T cells and $CD4^+$ T cells, respectively (5). After presentation, capsid-specific cytotoxic $CD8^+$ T cells can eliminate transduced hepatocytes, and $CD4^+$ T cells can activate plasma cells (6) and trigger an anti-capsid humoral response. Corticosteroids and immunosuppression have been shown to control these responses. Double-stranded messenger RNA transcribed from the viral genome in the nucleus can induce RIG-I-like receptors (RLRs) and melanoma differentiation-associated protein 5 (MDA5) sensors, which trigger an interferon type I immune response. AAV genome sequence modifications could prevent the activation of innate immune sensors such as TLR9. Activation of intrinsic cellular protective measures can also lead to episomal DNA loss or silencing (8), and AAV genome engineering could prevent this loss. ssDNA, single-stranded DNA.

Innate immune responses can also be activated by viral nucleic acid sequences, particularly unmethylated cytosine–guanine dinucleotide (CpG) motifs, that are recognized by the endosomal TLR9 in Kupffer cells and dendritic cells[95,99]. Several studies have shown that minimizing CpG motifs in transgenes helps to reduce loss of transgene expression[104,105]. However, other expression cassette elements, such as promoters and regulators of transgene expression, are also rich in CpG motifs, making it difficult to entirely eliminate these immunostimulatory sequences.

Complement activation has also been observed upon treatment with a high dose of rAAV, although the mechanism is yet to be fully elucidated[106]. Most evidence indicates an immunoglobulin-mediated pathway, but this possibility does not rule out the involvement of alternative antibody-independent complement activation pathways[106].

Finally, vector preparations can include host cell protein impurities, unknown packaged DNA material, an undetermined ratio of full to empty capsids, and other undesired components[107–109]. These elements are potentially immunogenic and can affect the potency of rAAV-mediated gene therapy. Standardization of quality control and a deep understanding of the process to produce recombinant AAV will minimize such impurities and lead to the development of safer, more potent and longer-lasting gene therapy.

Adverse effects

Hepatotoxicity. The most commonly observed adverse events in clinical trials of rAAV gene therapy are elevated serum levels of liver enzymes and liver failure[110] (Table 2). These events have been detected not only with liver-targeted rAAVs[92] but also with rAAV treatments for neurodegenerative and musculoskeletal diseases such as spinal muscle atrophy (SMA)[111,112] and X-linked myotubular myopathy (XLMTM)[113]. For example, across five clinical trials of onasemnogene abeparvovec (rAAV9-mediated delivery of the *SMN1* gene to motor neurons) for SMA, more than one-third of participants experienced at least one adverse event related to liver toxicity, some of which were severe[111,112].

The mechanism that underlies the hepatotoxicity of rAAV treatment is unclear. Corticosteroids are effective as a prophylactic and acute treatment for rAAV-induced hepatotoxicity, supporting the hypothesis that the cause is immunological in nature and can be mitigated with immunosuppressive drugs[96]. However, this treatment was not enough to prevent four deaths in a clinical trial of a treatment for XLMTM[113]. Direct causality of hepatotoxicity has not been established but some evidence implicates a combination of pre-existing hepatobiliary complications and high doses of rAAV[113]. The facts that the patients did not respond to immunosuppressive drugs in this case and that no notable inflammatory infiltrate was observed in their livers point towards hepatotoxicity caused by the vector or transgene rather than by an immune-mediated response. Additional information from the trial in XLMTM will be extremely valuable for understanding the mechanism involved in the patients' deaths and improving the safety of AAV-based clinical trials.

Hepatotoxicity in rAAV gene therapy has also been observed in animal studies. For instance, transient increases in liver enzymes and minimal to moderate histopathological findings were observed in the livers of neonatal wild-type mice that received onasemnogene abeparvovec at doses >1.1×10^{14} vg/kg (ref. [111]). Moreover, acute liver failure, thrombocytopenia and coagulopathy occurred in healthy non-human primates that received several rAAV serotypes carrying the *SMN1* transgene at doses >1×10^{14} vg/kg (refs. [114,115]). However, the mechanism involved remains to be elucidated.

Toxicity due to complement activation. Another major complication that has been reported in clinical trials of rAAV gene therapy is thrombotic microangiopathy (TMA) due to complement activation upon systemic administration of high doses of rAAV[106,116,117] (Table 2). As a result of microvascular thrombosis that occurs during TMA, damage

Table 2 | Toxicity associated with rAAV administration

Clinical observation	Severity	Mechanism	Mitigation strategies	Refs.
Transaminase elevation 3–6 weeks after rAAV administration	CTCAE grade 1–2 if ALT and AST levels are ≥ULN and ≤5× ULN[a]; CTCAE grade 3–4 if ALT and AST levels are >5× ULN	T cell immune response against the AAV capsid	Corticosteroid treatment	93,124
Transaminase elevation 3–6 weeks after rAAV administration, refractory to corticosteroid treatment	CTCAE grade 1–2 if ALT and AST levels are ≥ULN and ≤5× ULN[a];CTCAE grade 3–4 if ALT and AST levels are >5× ULN	T cell immune response against the AAV capsid associated with high CpG motif content that induced liver inflammation	Immunosuppressive drugs that target T cells; codon optimization in transgene to reduce CpG motif content	124
Hepatic failure	Severe, grade 3–4	Inflammatory immune response	Increase vector potency and specificity to decrease vector dose	124
Thrombotic microangiopathy	Severe, grade not specified by the CTCAE	Complement activation	Administration of eculizumab and C1 esterase inhibitor	124
Neurotoxicity	Not reported in humans	Inflammatory response in dorsal root ganglia	Design vector to avoid targeting of dorsal root ganglia	124
Genotoxicity[b]	Severe, grade not specified by the CTCAE	Integration of AAV vector sequences into the host genome	Reduce or eliminate gene expression drivers or regulatory elements from the AAV genome	124,201
Muscle weakness with variable cardiac involvement	Severe, grade not specified by the CTCAE	T cell immune response to the expressed transgene protein associated with the patient's genotype	Immunosuppressive and supportive therapies	124,253

AAV, adeno-associated virus; ALT, alanine transaminase; AST, aspartate transaminase; CpG, cytosine–guanine dinucleotide; CTCAE, NIH Common Terminology Criteria for Adverse Events; rAAV, recombinant AAV; ULN, upper limit of normal. [a]If baseline was normal. [b]Clinical cases reported with retroviral and lentiviral vectors; AAV cases reported in preclinical studies only thus far.

is caused to arterioles and capillaries[115,118]. Clinical signs include haemolytic anaemia, acute kidney injury and thrombocytopenia[118]. TMA had previously been described in preclinical studies but not in association with the clinical signs observed in patients[118].

The underlying mechanism of TMA after rAAV administration is unclear. Studies of human blood samples have shown a correlation between the presence of AAV-specific immunoglobulins and complement activation, which points to classical complement pathway activation as the culprit[119]. However, the classical pathway is only one of the three known complement pathways, and the other two — the lectin and the alternative pathways — can also be activated by viruses[120]. Therefore, further investigation of the molecular mechanisms of complement activation and TMA induction by rAAVs is necessary.

Cases of TMA after administration of therapeutic rAAVs were first reported in patients with SMA and Duchenne muscular dystrophy[121]. The effect might have been dose dependent as low doses of rAAV do not seem to activate the complement cascade[117]. In addition, one of the patients with SMA also carried a variant in the gene that encodes complement factor I that might have conferred a genetic predisposition to subclinical complement regulation insufficiency. TMA also occurred in two participants in a phase I–II clinical trial in children with MMA who received an rAAV LK03 serotype at a dose of 5×10^{13} vg/kg (ref. [122]). As a result, the trial was placed on clinical hold for several weeks. In this study, seronegativity for neutralizing antibodies against LK03 was a requirement for patient enrolment[123], indicating either that complement activation occurred via non-classical pathways or that pre-screening was not sufficient to prevent enrolment of patients who were at risk of developing antibody-mediated complement activation. The hold was lifted once safety measures were in place, including frequent testing for complement activation and use of a complement inhibitor.

In response to the reported cases of TMA, the FDA is monitoring cases of gene therapy toxicity closely. To date, the proposed risk mitigation plan includes prophylactic (off-label) use of the complement C5 antibody eculizumab and a C1 esterase inhibitor, higher daily doses of prednisolone than the dose prescribed for the first patients enroled in the trial, and modification of manufacturing processes to reduce empty capsids in rAAV vector preparations[110,124] (Box 2).

Neurotoxicity. Neurotoxicity has also been reported as a complication of rAAV gene therapy[125] (Table 2). Clinical data are limited to two case reports that included autopsy data[110], but evidence in non-human primates indicates degeneration of the primary sensory neurons in the dorsal root ganglia upon intrathecal or, to a lesser degree, systemic intravenous administration of rAAV at doses $>1 \times 10^{13}$ vg/kg (ref. [125]). These findings are relevant to liver-targeted rAAV gene therapy because intravenous administration is the preferred route. Dorsal root ganglia toxicity has been associated with the presence of vector genomes and capsid antigens that trigger an inflammatory response[126]; therefore, reducing non-specific rAAV transduction and transcription by using liver-specific capsids, promoters and regulatory elements could help to limit neurotoxicity (Box 2).

Strategies to mitigate adverse events. The implementation of some broad strategies to improve the safety profile of rAAV-based therapies could provide a roadmap for rAAV development (Table 2 and Box 2). Most of the observed adverse effects are associated with high doses and transduction of off-target tissues or cell types. Consequently, considerable effort is being made to develop optimized capsids to improve the efficiency and specificity of rAAV targeting so that lower doses can be used and the risk of toxicity lowered[127]. This approach could also reduce immune responses, minimizing the need for immunosuppression. In addition, improving the quality of the vector product by, for example, eliminating empty capsids and capsids that contain truncated genomes, could greatly improve the quality of treatment. Finally, the recombinant genome of rAAVs could be designed to avoid unwanted toxicities; for example, use of tissue-specific promoters could avoid transgene expression in off-target tissues[128] and the reduction of unmethylated CpG motifs to prevent TLR9 activation could minimize innate immune responses.

Box 2

Approaches to improve the safety of rAAVs in liver disease

- Develop recombinant adeno-associated virus (rAAV) vectors that target the liver more specifically to reduce the dose required.
- Standardize product quality and quality controls to detect and eliminate impurities from rAAV preparations and reduce unwanted toxicity.
- During clinical trial design, consider not only the dose as viral genomes per kilogram but also the total viral genomes administered to paediatric patients and patients with pre-existing liver conditions as a means of foreseeing risks of adverse effects and the use of additional prophylactic measures.
- Develop treatments to remove or block rAAV-specific antibodies and to prevent or mitigate complement activation.
- Reduce the content of immunostimulatory and potentially genotoxic elements in the therapeutic vector genome.
- Use preclinical disease models that mimic human pathology as closely as possible to determine their safety and efficacy, identify potential adverse effects, and develop mitigation plans.

Loss of rAAV genomes

AAV genomes mainly remain episomal in transduced cells. Though this has the benefit of limiting the risk of insertional mutagenesis, it is an important limitation of AAV-mediated gene therapy[32–35]. In contrast to integrative vectors, episomal AAV genomes are prone to a dilution effect during natural cell division, resulting in a loss of therapeutic effect. This limitation would be expected to be particularly relevant in young patients, in whom the rate of hepatocyte division is relatively high. However, ^{14}C dating of human hepatocytes from donors of different ages revealed continual renewal of hepatocytes regardless of the age of the liver and determined that the average age of all hepatocytes is only 3 years[129], suggesting that rAAV genome loss in dividing cells would not be restricted to children or patients with higher hepatocyte turnover due to liver damage but could affect all patients.

One proposed strategy to prevent the loss of recombinant genomes is the use of scaffold matrix attachment region sequences in the rAAV genome as these sequences induce its replication in conjunction with the cellular DNA[130]. Alternatively, strategies that permanently modify the genome, such as gene editing and the use of

integrative lentiviral vectors to insert DNA into the genome, could offer more lasting solutions[131–133]. Possible gene-editing strategies include non-homologous end-joining repair[134], homology-directed repair (HDR)[135–137], homology-independent targeted integration (HITI)[138], base editing[139], prime editing[23,140] and nuclease-free homologous recombination[128] (Fig. 3). Most of these strategies exploit the adaptability and potency of CRISPR technology (reviewed in detail elsewhere[141]).

rAAVs have been used as vectors for HDR, base editing and prime editing in proof-of-concept and preclinical studies with positive results[142–145]. For example, base editors delivered via rAAV8 to the liver in a mouse model of phenylketonuria corrected the *Pah* gene in up to 63% of cells and restored PAH enzyme activity[144]. However, approaches that produce more transient expression of DNA-editing machinery, such as lipid nanoparticle-mediated delivery of mRNA[134], could mitigate some safety concerns, but rAAVs are the best vectors for delivery of DNA templates for HDR and HITI, where delivery to the nucleus is required.

Preclinical studies of HDR strategies for permanent correction of disease-causing mutations have demonstrated very low efficiency unless corrected hepatocytes benefit from selective pressure, whereby only corrected hepatocytes have a selective advantage, survive and proliferate to repopulate the liver[142,143], or the treatment is administered in newborn mice[135–137]. Studies in mice and non-human primates have shown that the efficiency of gene editing is higher with HITI than with HDR[146,147]. HITI makes use of the non-homologous end-joining DNA repair pathway to knock in donor DNA and occurs both in dividing and non-dividing cells, which is of great interest for liver applications. More preclinical data are needed to confirm its potential for the treatment of inherited liver disorders and to evaluate its long-term safety and efficacy.

Nuclease-free homologous recombination involves the delivery of an rAAV that contains a promoterless therapeutic expression cassette flanked by DNA sequences that match or are homologous to the target sequence of the patient's genome. Therefore, the therapeutic transgene gets integrated into the genome in the absence of targeted nuclease activity (Fig. 3). The efficiency of this strategy in the absence of a double-strand DNA break is very low[128]; therefore, integration is targeted to the albumin locus such that transgene expression is regulated by the albumin promoter, which leads to potent transgene expression. Importantly, the system is designed to integrate the transgene without disrupting the expression of albumin[128]. This approach avoids the safety concerns inherent to nuclease-based strategies and, in theory, reduces the risks associated with random insertion as the transgene lacks its own promoter. However, the low editing efficiency limits the range of diseases that can be treated with this strategy[128,148–150]. Nevertheless, a phase I–II clinical trial of nuclease-free homologous recombination for the treatment of MMA is in progress[123].

Lentiviral vectors provide an alternative to gene editing for permanent modification of the genome. The therapeutic efficacy of lentiviral vectors has been tested in mouse models of various diseases (reviewed elsewhere[36]), including haemophilia B. Self-inactivating lentiviral vectors that lack long terminal repeat promoter elements have been developed to reduce the risk of insertional mutagenesis, and stable expression of factor IX in the livers of adult dogs and mice with haemophilia B has been achieved with such vectors[151]. Expression of recombinant factor IX was detected for >1 year in two of three dogs in this study, and episodes of spontaneous bleeding were reduced or eliminated in all three[151]. However, unwanted transduction of resident antigen-presenting cells in the liver led to suboptimal efficacy and acute toxicity — dogs that received the lentiviral vector developed a fever and mild transient hepatocellular toxicity accompanied by an increase in inflammatory cytokines.

Use of systemic administration of lentiviral vectors for gene therapy in liver diseases could increase in the future owing to the ongoing development of new hepatotropic lentiviral vector pseudotypes that avoid effects on off-target cell types. Preclinical studies with these vectors indicate efficacy, especially in neonatal animals, but more long-term studies are needed to address the intrinsic risk of insertional mutagenesis[152].

Limited translation from preclinical models

Translation of successful liver transduction and long-term transgene expression from small animal models to larger animals, such as non-human primates, and to patients has become one of the main challenges in the development of rAAV-mediated gene therapy[90,153,154]. For example, work in preclinical animal models did not predict the loss of recombinant protein expression due to $CD8^+$ T cell activation that was observed in patients in the trial of gene therapy for haemophilia B[93,155], teaching us a valuable lesson about the deficiencies of the preclinical models[90,153,154].

Similarly, the translation of dosage is not straightforward. For example, systemic administration of rAAV5 or rAAV8 at doses of 2×10^{11} to 5×10^{12} vg/kg had therapeutic efficacy in mouse models of AIP[3], Crigler–Najjar syndrome[155] and OTC deficiency[2] but, in non-human primates, the same range of doses of these two serotypes led to lower liver transduction and transgene expression at similar study endpoints[2,156]. Furthermore, in patients with AIP, use of higher doses than those that provided the full therapeutic effect in mice did not improve biochemical biomarkers of the disease[157] (Table 1). Whether the lower level of viral genome copies per cell in non-human primates compared with mice results from less efficient liver transduction, loss of viral genomes over time or a combination of both remains unclear. In a study in which non-human primates were administered a rAAV8 vector that expressed OTC at a dose of 1×10^{13} vg/kg, loss of transduced hepatocytes was seen between day 28 and day 140 (ref. [156]), suggesting that loss of transgene expression contributes to lack of efficacy. However, this outcome could be serotype dependent as administration of a serotype LK03 rAAV that expressed OTC at a dose of 2×10^{13} vg/kg in non-human primates resulted in sustained liver transduction and transgene expression for >180 days[158].

In addition to discrepancies in transduction efficiency between species, rAAVs transduce the liver in different patterns in mice, dogs and non-human primates[159]. For instance, in mice, rAAV8 transduces hepatocytes near the central veins more efficiently than those in the periportal region, whereas the reverse is true in non-human primates[159]. Furthermore, evidence that transduction occurs in a random pattern in infant macaques and newborn mice[159] suggests that the distribution pattern depends on the age at treatment and the corresponding liver architecture. Differences in transduction patterns are of foremost importance in metabolic liver diseases because there is a gradient of metabolic activity between periportal and central vein areas[1,160]. For instance, urea synthesis is greater in the periportal area[161], and therefore gene therapy mediated by rAAVs that target periportal hepatocytes is likely to be most efficient for restoring metabolic activity in urea cycle disorders. Understanding the reasons for the inverse zonation of hepatocyte transduction between mice and non-human primates could lead to better capsid design that improves the translatability of results from mouse studies.

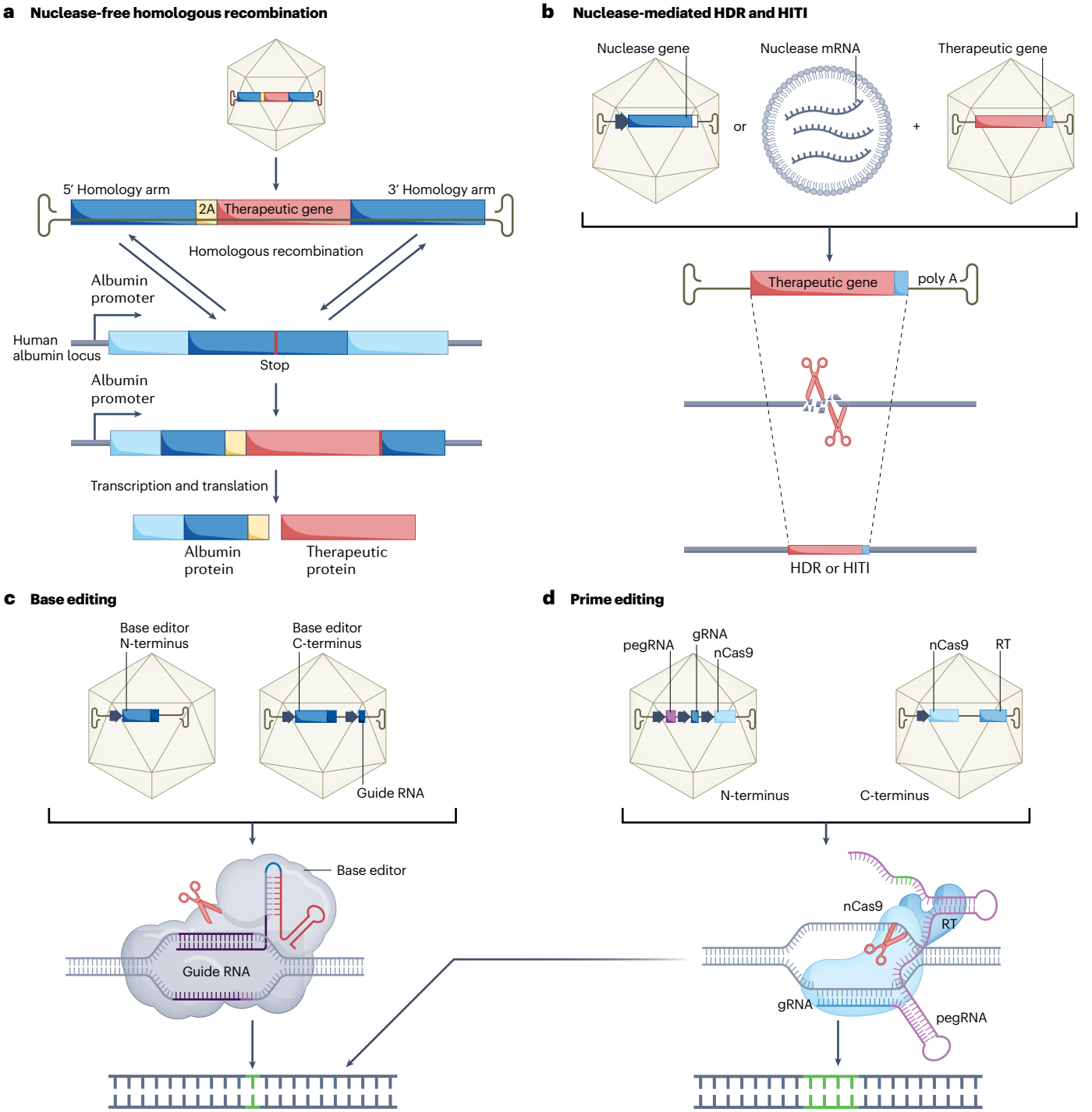

Fig. 3 | Strategies for liver genome editing with AAV vectors. a, Nuclease-free homologous recombination involves use of a recombinant adeno-associated virus (AAV) genome that contains the therapeutic gene flanked by homology arms that recombine into the albumin locus for target-specific insertion of the therapeutic gene. This strategy exploits the potent albumin transcription promoter to produce the therapeutic protein without affecting albumin expression. **b**, Nuclease-mediated homology-directed repair (HDR) and homology-independent targeted insertion (HITI) depend on delivery of the genetic material that encodes the nuclease in an AAV vector or lipid nanoparticle, together with the AAV that carries the therapeutic gene. The nuclease cuts the DNA to enable insertion of the therapeutic gene. **c**, Base editors are too large to fit in a single AAV but can be split between two AAVs. When expressed in liver cells, these editors induce sequence-specific single nucleotide changes to correct disease-causing point mutations. **d**, Like base editors, prime editors must be delivered via two AAV vectors and can be used to correct point mutations or insert or delete DNA fragments.

Development of new, more relevant animal models, such as humanized liver chimeric mice[162,163] and whole-organ explants[164], is essential for improving the translation of preclinical data to patients. For example, the engineered human-hepatotropic capsid LK03 was developed using humanized liver chimeric mice, and transduction with this capsid is more efficient than with AAV8 in human cells[162] and in whole human liver explants[164]. This improvement has been confirmed in a clinical trial in haemophilia A[165], in which use of the LK03 capsid to deliver the human coagulation factor VIII enabled a lower dose to be administered and led to a longer duration of transgene expression than did use of AAV5 or AAV6 (ref. [166]). Enrolment and screening for a clinical trial of AAV8 with the same transgene was suspended in August 2020 after an assessment of interim data[167] — lack of efficacy was the main decision driver. Results after 1 year of follow-up showed an elevation of factor IX activity, from 20% to 25%, in only one of eight participants. The lack of sustained transgene expression was associated with the CpG content of the construct. Ultimately, outcomes from well-designed clinical trials will determine the best approaches and doses for gene therapy to treat liver disorders.

Genotoxicity

Integration and oncogenicity of wild-type AAV. Evidence that the AAV genome can integrate into the human genome first came from studies of human cells with latent AAV infection in 1980 (ref. [168]). Later studies in the 1990s showed that wild-type AAV2 can integrate at a specific site in the chromosome 19q13.3–qter region of the human genome; this site is known as AAV integration site 1 (AAVS1)[169,170], also referred to as an AAV safe harbour site (Fig. 4). AAVS1 contains sequences that are very similar to the recognition site in ITRs for the Rep68 and Rep78 proteins that initiate replication, and these sequences enable binding of Rep and subsequent integration of the AAV genome[171]. Advanced sequencing and integration site analysis has shown that the wild-type AAV2 genome integrates into the AAVS1 locus and other genomic regions that contain similar Rep-binding sites[172]. Most integration events result in only partial integration of the AAV genome[173]. Insights into the integration mechanism have been exploited to design Rep-guided targeted integration strategies at the AAVS1 locus[174].

A study published in 2015 indicated an association between wild-type AAV2 integration and oncogene transactivation in hepatocellular carcinoma (HCC) tissue samples[173] (Fig. 4). In 10 of 11 samples, the AAV2 3′ ITR and upstream regions of variable length were inserted. These sequences contain liver-specific enhancers that enable transcription factor binding and activation of gene expression[175]. Most of the patients that the samples originated from did not have pre-existing conditions, such as cirrhosis, or any other risk factors for HCC[173]; therefore, the investigators concluded that integration of wild-type AAV2 DNA was directly associated with the development of HCC. In a subsequent study, integration of AAV2 and AAV2–AAV13 hybrids was seen in less than 10% of tumour samples[176], and other studies have demonstrated wild-type AAV2 genome integration in HCC tissue samples in smaller cohorts or at lower frequencies[177,178].

These findings raised considerable concerns about the safety of rAAV-based gene therapy. However, substantial uncertainties remain about the mechanism of oncogenicity and whether rAAVs will produce similar outcomes to those observed with wild-type AAVs[179,180]. Moreover, minimal to no evidence of oncogenic AAV genome integration in association with HCC or cholangiocarcinoma was found in a cohort of Asian patients[181], and searches of The Cancer Genome

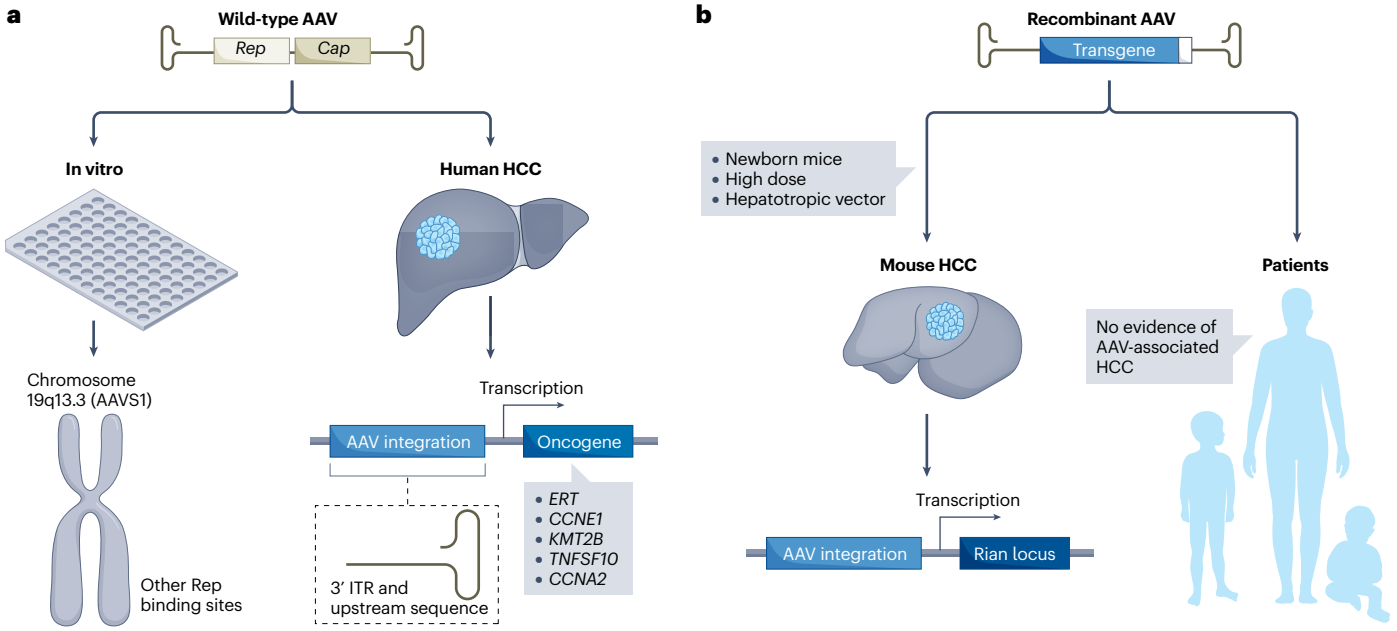

Fig. 4 | Wild-type and recombinant AAV liver oncogenicity findings in preclinical models and in humans. a, In vitro studies have shown that wild-type adeno-associated virus (AAV) can integrate into chromosome 19q13.3 at a site known as AAV integration site 1 (AAVS1) and other Rep-binding sites (left). Evidence also suggests that AAV integration events can activate various oncogenes in humans with hepatocellular carcinoma (HCC) (right). **b**, Recombinant AAVs that are used for therapeutic purposes have been associated with oncogenic integration events in animals, primarily in newborn mice treated with high doses of the hepatotropic vector (left). However, no oncogenic recombinant AAV integration events have been reported in any clinical trial in humans, indicating the need for better preclinical models to assess the risk of insertional mutagenesis. ITR, inverted terminal repeat.

Atlas have identified no association between AAV genome integration and cancer[182–184].

Conversely, some data even suggest that AAVs can induce anti-tumour activity[185]. Specifically, various studies have suggested that AAV transduction reduces malignant transformation[186], selectively kills cells that lack p53 (ref. [187]) and correlates with a lower frequency of cervical cancer[188]. However, these observations have either been made in vitro and/or causality has not been demonstrated.

Insertional mutagenesis. Concerns over insertional oncogenesis have been paramount in the development of rAAV therapy[189] (Table 2). Initial in vitro studies suggested that integration of rAAV genomes was random and occurred via non-homologous recombination, preferentially at chromosomal breakage sites[190,191]. Integrated partial rAAV genomes have been identified but expression of the integrated transgene had been silenced through histone modification and chromatin condensation[190,192,193]. These findings supported previous suggestions that the episomal copies of rAAV genomes are responsible for long-term in vivo expression[194,195].

Early in vivo analyses in mice also confirmed that rAAV genomes can integrate into the liver in a non-homologous manner, with some preference for active genes and gene regulatory sequences, such as transcription start sites and CpG islands[196,197]. In these studies, plasmid rescue strategies were used to recover integrated rAAV genomes, a technological limitation that restricted the breadth of hits, but integration into regulatory sequences of cancer-related genes was flagged[197]. These findings were reproduced using a PCR-based method that does not rely on marker gene expression, selection or cell division, and can therefore identify rAAV integration sites in non-dividing cells without cell manipulations[198]. Nowadays, improved PCR-based methods are used to identify insertion sites[189]. However, discussion is ongoing about the suitability of the currently used methods to detect AAV integration, particularly considering that most of them are based on PCR amplification methods, which are biased due to uneven fragmentation of genomic DNA and the complexity of AAV rearrangements at the site of integration[189].

Several studies have been conducted specifically to determine whether rAAV genome integration increases the risk of HCC[199–204]. In one of these studies, only high doses (~10^{14} vg/kg) of the serotypes rAAV8 and rAAV9, which have a high capacity for transduction, were associated with an increased risk of HCC in newborn mice, whereas lower doses were not and rAAV2, which has a lower transduction efficiency, was not associated with HCC at any dose, suggesting that the rAAV DNA construct and the amount of vector that reaches hepatocytes are both relevant[202]. However, the applicability of such results to patients is unclear because studies have been conducted in experimental systems that are designed to interrogate integration and/or tumour risk such as rAAV treatment in newborn mice[200–202,205,206], in tumour-prone or liver regeneration mouse models[204,207], or in liver injury[203]. Indeed, in one of these studies, HCC was purposely induced by directly integrating an rAAV vector into the *Rian* locus[203], which is not present in the human genome[201,202]. Furthermore, the associations between rAAV genome integration and the risk of HCC in these models have not been reproduced in studies of healthy adult mice[208,209] (Fig. 4), which have also demonstrated that hepatocyte proliferation due to liver growth or liver injury is important in the mechanism of rAAV-induced oncogenesis.

In addition to an awareness that animal models are imperfect attempts to mimic scenarios faced in the clinic (for example, paediatric patients and adult patients with fatty liver disease or chronic liver viral infections), the suitability of these models for assessing the risk of tumorigenesis as a result of rAAV genome integration at all is uncertain because mice are widely thought to be more prone to developing HCC than humans. In addition, in many preclinical studies, untreated mice with the disease have very limited survival, such that the control arm that enables assessment of relative risk is missing, or the disease phenotype includes development of HCC, making the involvement of rAAV treatment in its pathogenesis difficult to determine. This type of uncertainty led to a clinical trial of gene therapy for phenylketonuria being put on hold in 2021 because mice that had received the treatment developed liver tumours. Six of seven mice that received the highest dose of the therapeutic rAAV vector (2×10^{14} vg/kg) had developed tumours (five adenomas and one HCC) at week 52 after vector administration[210] (Table 1). The translatability of these findings to humans is uncertain and remains under investigation.

The risk of insertional mutagenesis has been characterized in greater depth in mice than in larger animal models. In a long-term study in which dogs were treated with rAAV-mediated gene therapy for haemophilia A, no evidence of tumours or altered liver function was detected over the 10-year follow-up period[211]. However, analysis of rAAV genome integration in the liver samples from the dogs did reveal that 44% of integration events were in regions close to genes involved in cell growth. Clonal cell expansion was also detected in five of six animals, which might have been the cause of a gradual increase in expression of dog factor VIII that started 4 years after treatment in two animals[211]. These findings showed that rAAV genome integration in genomic regions associated with cell proliferation does seem to happen in dogs and could, therefore, trigger liver genotoxicity.

By contrast, analyses of samples from non-human primates and humans have demonstrated that the frequency of integration is very low, and no clonal expansion or preferential integration has been observed[212–214]. In a phase III clinical trial of etranacogene dezaparvovec for the treatment of haemophilia B, one participant developed HCC 1 year after treatment[215]. Analysis of tumour tissue from this patient showed that the frequency of rAAV integration was very low and no different from that in adjacent healthy tissue. Furthermore, the patient had other risk factors for HCC (hepatitis B, hepatitis C and a family history of cancer). On this basis, the investigators concluded that the cancer was highly unlikely to have arisen as a result of rAAV genome integration and the hold on the clinical trial was removed by the FDA a few months after the adverse event was reported[189,215]. This case further highlights the need to study the safety of high doses of rAAV vectors in patients with pre-existing liver conditions and/or a high risk of HCC. Acute treatment-related adverse events, such as inflammation, could accelerate HCC tumorigenesis in these patients.

Age at treatment could influence the risk of genotoxicity as integration events might be more likely in children owing to the proliferation of liver cells during natural growth. Preclinical safety studies of treatment in young animals, including infant non-human primates, could help to address this possibility. In one such study, no safety issues were identified with the treatment of infant non-human primates with rAAV9 during a follow-up period of 3 years and 9 months, although this might be too early to draw conclusions about clonal expansion or HCC[216].

Some evidence suggests that rAAV-mediated phenotypic correction of disorders that lead to HCC can actually prevent tumour formation in cells and organs that are successfully transduced by the vector[217] (Weber, N.D. et al., unpublished work). One possible mechanism for

this effect is reduced hepatocyte regeneration owing to a therapeutic effect. Nevertheless, the evidence that rAAV genome insertion could be associated with an increased risk of tumours means that this risk must always be accounted for and monitored carefully. However, besides the single patient in the phase III trial in haemophilia B, no other cases of HCC have been reported in association with rAAV gene therapy during >25 years of clinical studies in which >3,000 patients of different ages have received the treatment at various doses.

Risks with rAAV-mediated expression of genome editors. Therapies that modify host DNA introduce several safety concerns and the theoretical risk is compounded when rAAV is used to deliver the gene-editing machinery. Off-target DNA editing is a widely held concern, and episomal stabilization of the rAAV genome that carries the gene editor could increase the likelihood of off-target modification over time. Several analytical methods have been developed to identify such off-target effects[218]. Off-target analysis must be performed on the human genome and on a case-by-case basis for each target sequence; therefore, preclinical prediction of off-target effects must be conducted with in vitro studies of human cells, in non-human primates (with limitations) or in humanized liver models[134,218–220].

Strategies to mitigate genotoxicity. To address the potential for genotoxicity of rAAV gene therapies, preclinical characterization of the therapies in appropriate models is essential. In addition, methods that enable unbiased analysis of rAAV genome integration would improve estimates of the risk–benefit ratio[189].

One approach to reduce oncogenic integration of rAAV genomes is the use of vectors that do not include promoters and undergo targeted integration into safe genomic regions. Theoretically, this approach reduces the oncogenic risk in two ways[128]: first, the frequency of random integration events should be reduced owing to the targeting; second, the lack of a promoter enables the transgene to be expressed if integrated at its target site downstream of an endogenous promoter but reduces the likelihood that the gene will be expressed and induce runaway proliferation in the event of unintended integration near an oncogene[128]. However, these theoretical benefits are yet to be demonstrated and would not completely eliminate the risk of integration at unintended sites such as double-strand DNA breaks, homologous regions and palindromic sequences.

Another strategy is to reduce or eliminate promoter sequences in the vector genome as these are open DNA sequences that provide binding sites for transcription factors that increase expression. In general, if promoters are included, the use of mammalian promoter sequences is preferable to the use of viral promoters because their activity is better understood and controlled in the context of the human genome. In addition, cell-specific elements can be used to restrict the expression of the transgene to the target tissue. Promoters are known to stimulate gene expression over large distances as well as in trans[175,221–223], so their potential effects on genotoxicity must be fully understood before they are included in gene therapies.

Conclusion

Since the first clinical trial of gene therapy in 1990, the approach has traversed a long and complicated journey with many ups and downs. Therapeutic efficacy and safety have been demonstrated in many different diseases in animal models but relatively few have succeeded in the clinic to date. Gene therapy mediated by rAAV vectors has emerged as a real therapeutic option — four such therapies, including one targeting the liver, have been approved by the FDA or EMA, three are in pre-registration, and more are close to regulatory assessment.

rAAV-mediated gene therapy is particularly attractive for the treatment of inherited liver disorders and diseases in which hepatic protein expression is beneficial owing to the natural hepatotropism of AAV. Long-term therapeutic data obtained in phase III clinical trials of rAAV-mediated gene therapy for haemophilia A and B, together with encouraging results from phase I–II trials of therapy for OTC deficiency, GSD1a and Crigler–Najjar syndrome, suggest that multiple liver-targeted gene therapy products will gain market approval in the near future, paving the way for gene therapy to address other incurable diseases. However, several aspects need careful evaluation before clinical approval can be justified.

First, a possible toxic effect has been associated with high viral doses, particularly in patients with underlying liver disease as demonstrated in patients with XLMTM. Strategies to improve hepatic pathology before vector administration should be thoroughly explored, and preclinical studies of efficacy and safety in animals with disease phenotypes that resemble those of concern in patients are obligatory. Second, though evidence of rAAV genotoxicity comes from studies in animal models that might not accurately represent patient characteristics, the long-term consequences of the treatment must be closely monitored. Third, a major limitation of rAAV-mediated gene therapy for the treatment of inherited liver diseases is the loss of vector genomes upon cell division and the resulting loss of effect, a phenomenon that is particularly important in young patients. Strategies that enable vector re-administration are under development and will hopefully make re-treatment of young patients possible if the therapeutic effect wanes. Alternatively, the transience of the rAAV-mediated therapeutic effect could be solved by introducing permanent modifications into the patient's genome via gene editing. Fourth, we need to improve our understanding of the mechanisms involved in severe short-term and long-term adverse effects of rAAV-mediated gene therapy, such as acute life-threatening toxicity and insertional mutagenesis, and results from clinical trials are dictating new bench work and animal studies.

Ultimately, these efforts will lead to the development of safer and more efficient strategies for gene augmentation and gene editing. Given the proven efficacy and powerful versatility of liver-targeted gene therapy and the improvements that can be made with work to be done, there is strong optimism among researchers and clinicians alike that much of the envisioned potential of such therapy can become reality.

Published online: 16 January 2023

References

1. Schulze, R. J., Schott, M. B., Casey, C. A., Tuma, P. L. & McNiven, M. A. The cell biology of the hepatocyte: a membrane trafficking machine. *J. Cell Biol.* **218**, 2096–2112 (2019).
2. Wang, L. et al. AAV gene therapy corrects OTC deficiency and prevents liver fibrosis in aged OTC-knock out heterozygous mice. *Mol. Genet. Metab.* **120**, 299–305 (2017).
3. Unzu, C. et al. Sustained enzymatic correction by rAAV-mediated liver gene therapy protects against induced motor neuropathy in acute porphyria mice. *Mol. Ther.* **19**, 243–250 (2011).
4. Kaiser, R. A. et al. Use of an adeno-associated virus serotype Anc80 to provide durable cure of phenylketonuria in a mouse model. *J. Inherit. Metab. Dis.* **44**, 1369–1381 (2021).
5. Weber, N. D. et al. Gene therapy for progressive familial intrahepatic cholestasis type 3 in a clinically relevant mouse model. *Nat. Commun.* **10**, 5694 (2019).
6. Wojnarowska, F. The disfigured patient. *Practitioner* **232**, 1022–1025 (1988).
7. Puzzo, F. et al. Rescue of Pompe disease in mice by AAV-mediated liver delivery of secretable acid alpha-glucosidase. *Sci. Transl. Med.* https://doi.org/10.1126/scitranslmed.aam6375 (2017).
8. Ronzitti, G. et al. A translationally optimized AAV-UGT1A1 vector drives safe and long-lasting correction of Crigler-Najjar syndrome. *Mol. Ther. Methods Clin. Dev.* **3**, 16049 (2016).
In this study, in vitro and in vivo testing of non-coding and codon-optimized transgene sequences revealed a great deal about vector construct design and about producing long-lasting disease correction in multiple animal models.

9. Le Quellec, S. et al. Recombinant adeno-associated viral vectors expressing human coagulation FIX-E456H variant in Hemophilia B mice. *Thromb. Haemost.* **119**, 1956–1967 (2019).
10. McIntosh, J. et al. Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. *Blood* **121**, 3335–3344 (2013).
11. Friedmann, T. & Roblin, R. Gene therapy for human genetic disease? *Science* **175**, 949–955 (1972).
12. Jackson, D. A., Symons, R. H. & Berg, P. Biochemical method for inserting new genetic information into DNA of simian virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. 1972. *Biotechnology* **24**, 11–16 (1992).
13. Mertz, J. E. & Davis, R. W. Cleavage of DNA by R 1 restriction endonuclease generates cohesive ends. *Proc. Natl Acad. Sci. USA* **69**, 3370–3374 (1972).
14. Lobban, P. E. & Kaiser, A. D. Enzymatic end-to end joining of DNA molecules. *J. Mol. Biol.* **78**, 453–471 (1973).
15. Cohen, S. N., Chang, A. C., Boyer, H. W. & Helling, R. B. Construction of biologically functional bacterial plasmids in vitro. *Proc. Natl Acad. Sci. USA* **70**, 3240–3244 (1973).
16. Blaese, R. M. et al. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science* **270**, 475–480 (1995).
This study was a first-in-human gene therapy clinical trial, in which a patient with severe immunodeficiency received T cells modified with a retroviral vector carrying the therapeutic gene.
17. Shahryari, A. et al. Development and clinical translation of approved gene therapy products for genetic disorders. *Front. Genet.* **10**, 868 (2019).
18. Anguela, X. M. & High, K. A. Entering the modern era of gene therapy. *Annu. Rev. Med.* **70**, 273–288 (2019).
19. Zabaleta, N., Hommel, M., Salas, D. & Gonzalez-Aseguinolaza, G. Genetic-based approaches to inherited metabolic liver diseases. *Hum. Gene Ther.* **30**, 1190–1203 (2019).
20. Alkhouri, N. & Gawrieh, S. A perspective on RNA interference-based therapeutics for metabolic liver diseases. *Expert. Opin. Investig. Drugs* **30**, 237–244 (2021).
21. Doudna, J. A. The promise and challenge of therapeutic genome editing. *Nature* **578**, 229–236 (2020).
22. Rees, H. A. & Liu, D. R. Base editing: precision chemistry on the genome and transcriptome of living cells. *Nat. Rev. Genet.* **19**, 770–788 (2018).
23. Anzalone, A. V. et al. Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* **576**, 149–157 (2019).
24. CSL Behring. *FDA Accepts CSL Behring's Biologics License Application for Etranacogene Dezaparvovec for Priority Review* https://www.cslbehring.com/newsroom/2022/fda-bla-etranacogene-dezaparvovec (2022).
25. European Medicines Agency. *First Gene Therapy to Treat Severe Haemophilia A* https://www.ema.europa.eu/en/news/first-gene-therapy-treat-severe-haemophilia (2022).
26. Srivastava, A., Lusby, E. W. & Berns, K. I. Nucleotide sequence and organization of the adeno-associated virus 2 genome. *J. Virol.* **45**, 555–564 (1983).
27. Atchison, R. W., Casto, B. C. & Hammon, W. M. Adenovirus-associated defective virus particles. *Science* **149**, 754–756 (1965).
28. Gao, G. et al. Clades of Adeno-associated viruses are widely disseminated in human tissues. *J. Virol.* **78**, 6381–6388 (2004).
29. Shirley, J. L., de Jong, Y. P., Terhorst, C. & Herzog, R. W. Immune responses to viral gene therapy vectors. *Mol. Ther.* **28**, 709–722 (2020).
30. Mendell, J. R. et al. Current clinical applications of in vivo gene therapy with AAVs. *Mol. Ther.* **29**, 464–488 (2021).
31. Wang, D., Tai, P. W. L. & Gao, G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat. Rev. Drug Discov.* **18**, 358–378 (2019).
32. Samulski, R. J. & Muzyczka, N. AAV-mediated gene therapy for research and therapeutic purposes. *Annu. Rev. Virol.* **1**, 427–451 (2014).
33. Flotte, T. R., Afione, S. A. & Zeitlin, P. L. Adeno-associated virus vector gene expression occurs in nondividing cells in the absence of vector DNA integration. *Am. J. Respir. Cell Mol. Biol.* **11**, 517–521 (1994).
34. Vincent-Lacaze, N. et al. Structure of adeno-associated virus vector DNA following transduction of the skeletal muscle. *J. Virol.* **73**, 1949–1955 (1999).
35. Yang, J. et al. Concatamerization of adeno-associated virus circular genomes occurs through intermolecular recombination. *J. Virol.* **73**, 9468–9477 (1999).
36. Maestro, S., Weber, N. D., Zabaleta, N., Aldabe, R. & Gonzalez-Aseguinolaza, G. Novel vectors and approaches for gene therapy in liver diseases. *JHEP Rep.* **3**, 100300 (2021).
37. Gao, G., Vandenberghe, L. H. & Wilson, J. M. New recombinant serotypes of AAV vectors. *Curr. Gene Ther.* **5**, 285–297 (2005).
38. Tiegs, G. & Lohse, A. W. Immune tolerance: what is unique about the liver. *J. Autoimmun.* **34**, 1–6 (2010).
39. Keeler, G. D., Markusic, D. M. & Hoffman, B. E. Liver induced transgene tolerance with AAV vectors. *Cell Immunol.* **342**, 103728 (2019).
40. Crispe, I. N. Immune tolerance in liver disease. *Hepatology* **60**, 2109–2117 (2014).
41. Lerut, J. & Sanchez-Fueyo, A. An appraisal of tolerance in liver transplantation. *Am. J. Transpl.* **6**, 1774–1780 (2006).
42. Protzer, U., Maini, M. K. & Knolle, P. A. Living in the liver: hepatic infections. *Nat. Rev. Immunol.* **12**, 201–213 (2012).
43. Crispe, I. N. Hepatic T cells and liver tolerance. *Nat. Rev. Immunol.* **3**, 51–62 (2003).
44. Zhang, P. et al. Immunodominant liver-specific expression suppresses transgene-directed immune responses in murine pompe disease. *Hum. Gene Ther.* **23**, 460–472 (2012).
45. Wu, Z. et al. Optimization of self-complementary AAV vectors for liver-directed expression results in sustained correction of hemophilia B at low vector dose. *Mol. Ther.* **16**, 280–289 (2008).
46. Szafranska, K., Kruse, L. D., Holte, C. F., McCourt, P. & Zapotoczny, B. The whole story about fenestrations in LSEC. *Front. Physiol.* **12**, 735573 (2021).
47. Von Drygalski, A. et al. Etranacogene dezaparvovec (AMT-061 phase 2b): normal/near normal FIX activity and bleed cessation in hemophilia B. *Blood Adv.* **3**, 3241–3247 (2019).
48. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03569891 (2018).
49. US Food and Drug Administration. BLA approval letter for etranacogene dezaparvovec. *FDA* https://www.fda.gov/media/163466/download (2022).
50. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03861273 (2019).
51. Robinson, M. M. et al. Factor IX assay discrepancies in the setting of liver gene therapy using a hyperfunctional variant factor IX-Padua. *J. Thromb. Haemost.* **19**, 1212–1218 (2021).
52. Konkle, B. A. et al. Updated follow-up of the Alta study, a phase 1/2, open label, adaptive, dose-ranging study to assess the safety and tolerability of SB-525 gene therapy in adult patients with severe hemophilia A. *Blood* **134**, 2060 (2019).
53. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT04370054 (2020).
54. Pasi, K. J. et al. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. *N. Engl. J. Med.* **382**, 29–40 (2020).
Results from the clinical trial of roctavian, the first approved AAV-based gene therapy drug directed to the liver.
55. Pasi, K. J. et al. Persistence of haemostatic response following gene therapy with valoctocogene roxaparvovec in severe haemophilia A. *Haemophilia* **27**, 947–956 (2021).
56. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT04323098 (2020).
57. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03370913 (2017).
58. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03392974 (2018).
59. Kassim, S. H. et al. Gene therapy in a humanized mouse model of familial hypercholesterolemia leads to marked regression of atherosclerosis. *PLoS ONE* **5**, e13424 (2010).
60. Murillo, O. et al. Liver expression of a MiniATP7B gene results in long-term restoration of copper homeostasis in a Wilson disease model in mice. *Hepatology* **70**, 108–126 (2019).
61. Lee, Y. M. et al. Long-term safety and efficacy of AAV gene therapy in the canine model of glycogen storage disease type Ia. *J. Inherit. Metab. Dis.* **41**, 977–984 (2018).
62. Ilyinskii, P. O. et al. ImmTOR nanoparticles enhance AAV transgene expression after initial and repeat dosing in a mouse model of methylmalonic acidemia. *Mol. Ther. Methods Clin. Dev.* **22**, 279–292 (2021).
63. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT02991144 (2016).
64. Geberhiwot, T. in ESGCT Collaborative Virtual Congress Vol. A1-A152 (European Society of Gene & Cell Therapy, 2021).
65. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03952156 (2019).
66. Bodamer, O. 2021 ACMG Annual Clinical Genetics Meeting. *Mol. Gen. Metabol.* **132**, S1–S382 (2021).
67. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03517085 (2018).
68. Couce Pico, M. L. in ESGCT Collaborative Virtual Congress Vol. 32 A1-A152 (ESGCT, 2021).
69. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03466463 (2018).
70. D'Antiga, L. ESGCT Collaborative Virtual Congress 19–22 October 2021 Abstracts. *Hum. Gene Ther.* **32**, A1–A152 (2021).
71. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT05345171 (2022).
72. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT05139316 (2021).
73. Verdera, H. C., Kuranda, K. & Mingozzi, F. AAV vector immunogenicity in humans: a long journey to successful gene transfer. *Mol. Ther.* **28**, 723–746 (2020).
74. Li, C. et al. Neutralizing antibodies against adeno-associated virus examined prospectively in pediatric patients with hemophilia. *Gene Ther.* **19**, 288–294 (2012).
75. Perocheau, D. P. et al. Age-related seroprevalence of antibodies against AAV-LK03 in a UK population cohort. *Hum. Gene Ther.* **30**, 79–87 (2019).
76. Boutin, S. et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum. Gene Ther.* **21**, 704–712 (2010).
77. Calcedo, R. & Wilson, J. M. AAV natural infection induces broad cross-neutralizing antibody responses to multiple AAV serotypes in chimpanzees. *Hum. Gene Ther. Clin. Dev.* **27**, 79–82 (2016).
78. Scallan, C. D. et al. Human immunoglobulin inhibits liver transduction by AAV vectors at low AAV2 neutralizing titers in SCID mice. *Blood* **107**, 1810–1817 (2006).

79. Mingozzi, F. & High, K. A. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. *Blood* **122**, 23–36 (2013).
80. Chicoine, L. G. et al. Plasmapheresis eliminates the negative impact of AAV antibodies on microdystrophin gene expression following vascular delivery. *Mol. Ther.* **22**, 338–347 (2014).
81. Salas, D. et al. Immunoadsorption enables successful rAAV5-mediated repeated hepatic gene delivery in nonhuman primates. *Blood Adv.* **3**, 2632–2641 (2019).
82. Mingozzi, F. et al. Overcoming preexisting humoral immunity to AAV using capsid decoys. *Sci. Transl. Med.* **5**, 194ra192 (2013).
83. Bertin, B. et al. Capsid-specific removal of circulating antibodies to adeno-associated virus vectors. *Sci. Rep.* **10**, 864 (2020).
84. Leborgne, C. et al. IgG-cleaving endopeptidase enables in vivo gene therapy in the presence of anti-AAV neutralizing antibodies. *Nat. Med.* **26**, 1096–1101 (2020).
This study demonstrated that the use of bacterial proteins with capacity to cleave human IgG might open the door for the treatment of patients with antibodies against AAV, currently excluded from gene therapy clinical trials.
85. von Pawel-Rammingen, U., Johansson, B. P. & Bjorck, L. IdeS, a novel streptococcal cysteine proteinase with unique specificity for immunoglobulin G. *EMBO J.* **21**, 1607–1615 (2002).
86. Elmore, Z. C., Oh, D. K., Simon, E. E., Fanous, M. M. & Asokan, A. Rescuing AAV gene transfer from neutralizing antibodies with an IgG-degrading enzyme. *JCI Insight* **5**, e139881 (2020).
87. Winstedt, L. et al. Complete removal of extracellular IgG antibodies in a randomized dose-escalation phase I study with the bacterial enzyme IdeS — a novel therapeutic opportunity. *PLoS ONE* **10**, e0132011 (2015).
88. Tseng, Y. S. & Agbandje-McKenna, M. Mapping the AAV capsid host antibody response toward the development of second generation gene delivery vectors. *Front. Immunol.* **5**, 9 (2014).
89. Meliani, A. et al. Antigen-selective modulation of AAV immunogenicity with tolerogenic rapamycin nanoparticles enables successful vector re-administration. *Nat. Commun.* **9**, 4098 (2018).
This study showed that the use of lipid nanoparticles containing rapamycin can prevent antibody formation after the first dose of a viral vector, allowing vector re-administration.
90. Mingozzi, F. et al. $CD8^+$ T-cell responses to adeno-associated virus capsid in humans. *Nat. Med.* **13**, 419–422 (2007).
91. Li, H. et al. Capsid-specific T-cell responses to natural infections with adeno-associated viruses in humans differ from those of nonhuman primates. *Mol. Ther.* **19**, 2021–2030 (2011).
92. Manno, C. S. et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat. Med.* **12**, 342–347 (2006).
The first AAV-mediated clinical trial targeting the liver for the correction of haemophilia B. This trial highlighted the importance of controlling the cellular immune response against the capsid to achieve sustained transgene expression.
93. Nathwani, A. C. et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N. Engl. J. Med.* **365**, 2357–2365 (2011).
This study demonstrated long-term factor IX expression after AAV injection in several patients thanks to the use of corticoids to block anti-AAV T cell responses and prevent the disappearance of the transduced cells.
94. George, L. A. et al. Hemophilia B gene therapy with a high-specific-activity factor IX variant. *N. Engl. J. Med.* **377**, 2215–2227 (2017).
95. Konkle, B. A. et al. BAX 335 hemophilia B gene therapy clinical trial results: potential impact of CpG sequences on gene expression. *Blood* **137**, 763–774 (2021).
96. Chai, Z. et al. Optimization of dexamethasone administration for maintaining global transduction efficacy of adeno-associated virus serotype 9. *Hum. Gene Ther.* **30**, 829–840 (2019).
97. Schroeder, H. 25th Annual Meeting of the American Society of Gene & Cell Therapy. *Mol. Ther.* **30** (Suppl. 1), 1–592 (2022).
98. Hosel, M. et al. Toll-like receptor 2-mediated innate immune response in human nonparenchymal liver cells toward adeno-associated viral vectors. *Hepatology* **55**, 287–297 (2012).
99. Zhu, J., Huang, X. & Yang, Y. The TLR9-MyD88 pathway is critical for adaptive immune responses to adeno-associated virus gene therapy vectors in mice. *J. Clin. Invest.* **119**, 2388–2398 (2009).
100. Rogers, G. L. et al. Unique roles of TLR9- and MyD88-dependent and -independent pathways in adaptive immune responses to AAV-mediated gene transfer. *J. Innate Immun.* **7**, 302–314 (2015).
101. Earley, L. F. et al. Adeno-associated virus serotype-specific inverted terminal repeat sequence role in vector transgene expression. *Hum. Gene Ther.* **31**, 151–162 (2020).
102. Flotte, T. R. et al. Gene expression from adeno-associated virus vectors in airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **7**, 349–356 (1992).
103. Shao, W. et al. Double-stranded RNA innate immune response activation from long-term adeno-associated virus vector transduction. *JCI Insight* **3**, e120474 (2018).
104. Faust, S. M. et al. CpG-depleted adeno-associated virus vectors evade immune detection. *J. Clin. Invest.* **123**, 2994–3001 (2013).
105. Xiang, Z. et al. The effect of CpG sequences on capsid-specific $CD8^+$ T cell responses to AAV vector gene transfer. *Mol. Ther.* **28**, 771–783 (2020).
106. Flotte, T. R. Revisiting the "new" inflammatory toxicities of adeno-associated virus vectors. *Hum. Gene Ther.* **31**, 398–399 (2020).
107. Grieger, J. C., Soltys, S. M. & Samulski, R. J. Production of recombinant adeno-associated virus vectors using suspension HEK293 cells and continuous harvest of vector from the culture media for GMP FIX and FLT1 clinical vector. *Mol. Ther.* **24**, 287–297 (2016).
108. Schnodt, M. & Buning, H. Improving the quality of adeno-associated viral vector preparations: the challenge of product-related impurities. *Hum. Gene Ther. Methods* **28**, 101–108 (2017).
109. Pierson, E. E., Keifer, D. Z., Asokan, A. & Jarrold, M. F. Resolving adeno-associated viral particle diversity with charge detection mass spectrometry. *Anal. Chem.* **88**, 6718–6725 (2016).
110. Mullard, A. Gene therapy community grapples with toxicity issues, as pipeline matures. *Nat. Rev. Drug Discov.* **20**, 804–805 (2021).
111. Chand, D. et al. Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy. *J. Hepatol.* **74**, 560–566 (2021).
In this study, systemic administration of high doses of recombinant AAV vector to treat a neuromuscular disease resulted in severe liver damage.
112. Feldman, A. G. et al. Subacute liver failure following gene replacement therapy for spinal muscular atrophy type 1. *J. Pediatr.* **225**, 252–258.e1 (2020).
113. Morales, L., Gambhir, Y., Bennett, J. & Stedman, H. H. Broader implications of progressive liver dysfunction and lethal sepsis in two boys following systemic high-dose AAV. *Mol. Ther.* **28**, 1753–1755 (2020).
114. Hinderer, C. et al. Severe toxicity in nonhuman primates and piglets following high-dose intravenous administration of an adeno-associated virus vector expressing human SMN. *Hum. Gene Ther.* **29**, 285–298 (2018).
115. Palazzi, X. et al. Biodistribution and tolerability of AAV-PHP.B-CBh-SMN1 in Wistar Han rats and cynomolgus macaques reveal different toxicologic profiles. *Hum. Gene Ther.* **33**, 175–187 (2022).
116. Chand, D. H. et al. Thrombotic microangiopathy following onasemnogene abeparvovec for spinal muscular atrophy: a case series. *J. Pediatr.* **231**, 265–268 (2021).
117. Muhuri, M. et al. Overcoming innate immune barriers that impede AAV gene therapy vectors. *J. Clin. Invest.* https://doi.org/10.1172/JCI143780 (2021).
118. Zaiss, A. K. et al. Complement is an essential component of the immune response to adeno-associated virus vectors. *J. Virol.* **82**, 2727–2740 (2008).
119. Smith, C. J. et al. Pre-existing humoral immunity and complement pathway contribute to immunogenicity of adeno-associated virus (AAV) vector in human blood. *Front. Immunol.* **13**, 999021 (2022).
120. Agrawal, P. et al. The imitation game: a viral strategy to subvert the complement system. *FEBS Lett.* **594**, 2518–2542 (2020).
121. Guillou, J. et al. Fatal thrombotic microangiopathy case following adeno-associated viral SMN gene therapy. *Blood Adv.* https://doi.org/10.1182/bloodadvances.2021006419 (2022).
122. PR Newswire. *LogicBio Therapeutics Provides Update on LB-001 Clinical Development Program* https://www.prnewswire.com/news-releases/logicbio-therapeutics-provides-update-on-lb-001-clinical-development-program-301473512.html (2022).
123. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT04581785 (2020).
124. FDA. *Cellular, Tissue, and Gene Therapies Advisory Committee September 2, 2021 Meeting Presentation- Toxicity Risks of Adeno-associated Virus (AAV) Vectors for Gene Therapy (GT)* www.fda.gov (2021).
125. Hordeaux, J. et al. Adeno-associated virus-induced dorsal root ganglion pathology. *Hum. Gene Ther.* **31**, 808–818 (2020).
126. Hordeaux, J. et al. MicroRNA-mediated inhibition of transgene expression reduces dorsal root ganglion toxicity by AAV vectors in primates. *Sci. Transl. Med.* **12**, eaba9188 (2020).
127. Zolotukhin, S. & Vandenberghe, L. H. AAV capsid design: a Goldilocks challenge. *Trends Mol. Med.* **28**, 183–193 (2022).
128. Barzel, A. et al. Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. *Nature* **517**, 360–364 (2015).
129. Heinke, P. et al. Diploid hepatocytes drive physiological liver renewal in adult humans. *Cell Syst.* **13**, 499–507.e12 (2022).
130. Hagedorn, C. et al. S/MAR element facilitates episomal long-term persistence of adeno-associated virus vector genomes in proliferating cells. *Hum. Gene Ther.* **28**, 1169–1179 (2017).
131. Jinek, M. et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816–821 (2012).
132. Follenzi, A., Sabatino, G., Lombardo, A., Boccaccio, C. & Naldini, L. Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. *Hum. Gene Ther.* **13**, 243–260 (2002).
133. Milani, M. et al. Liver-directed lentiviral gene therapy corrects hemophilia A mice and achieves normal-range factor VIII activity in non-human primates. *Nat. Commun.* **13**, 2454 (2022).
134. Gillmore, J. D. et al. CRISPR-Cas9 in vivo gene editing for transthyretin amyloidosis. *N. Engl. J. Med.* **385**, 493–502 (2021).
The first clinical trial in which CRISPR was used to target the liver for treatment of an inherited disorder.
135. Ohmori, T. et al. CRISPR/Cas9-mediated genome editing via postnatal administration of AAV vector cures haemophilia B mice. *Sci. Rep.* **7**, 4159 (2017).
136. Yang, Y. et al. A dual AAV system enables the Cas9-mediated correction of a metabolic liver disease in newborn mice. *Nat. Biotechnol.* **34**, 334–338 (2016).

137. Richards, D. Y. et al. AAV-Mediated CRISPR/Cas9 gene editing in murine phenylketonuria. *Mol. Ther. Methods Clin. Dev.* **17**, 234–245 (2020).
138. Suzuki, K. et al. In vivo genome editing via CRISPR/Cas9 mediated homology-independent targeted integration. *Nature* **540**, 144–149 (2016).
139. Levy, J. M. et al. Cytosine and adenine base editing of the brain, liver, retina, heart and skeletal muscle of mice via adeno-associated viruses. *Nat. Biomed. Eng.* **4**, 97–110 (2020).
140. Awan, M. J. A., Ali, Z., Amin, I. & Mansoor, S. Twin prime editor: seamless repair without damage. *Trends Biotechnol.* **40**, 374–376 (2022).
141. Yang, L. & Chen, J. A tale of two moieties: rapidly evolving CRISPR/Cas-based genome editing. *Trends Biochem. Sci.* **45**, 874–888 (2020).
142. Yin, H. et al. Therapeutic genome editing by combined viral and non-viral delivery of CRISPR system components in vivo. *Nat. Biotechnol.* **34**, 328–333 (2016).
143. Krooss, S. A. et al. Ex vivo/in vivo gene editing in hepatocytes using "All-in-One" CRISPR-adeno-associated virus vectors with a self-linearizing repair template. *iScience* **23**, 100764 (2020).
144. Villiger, L. et al. Treatment of a metabolic liver disease by in vivo genome base editing in adult mice. *Nat. Med.* **24**, 1519–1525 (2018).
145. Liu, P. et al. Improved prime editors enable pathogenic allele correction and cancer modelling in adult mice. *Nat. Commun.* **12**, 2121 (2021).
146. Suzuki, K. & Izpisua Belmonte, J. C. In vivo genome editing via the HITI method as a tool for gene therapy. *J. Hum. Genet.* **63**, 157–164 (2018).
147. Intellia. *Intellia Therapeutics Achieves Normal Human Alpha-1 Antitrypsin Protein Levels in Non-Human Primates Through Targeted Gene Insertion for the Treatment of AAT Deficiency* https://ir.intelliatx.com/news-releases/news-release-details/intellia-therapeutics-achieves-normal-human-alpha-1-antitrypsin (2020).
148. Porro, F. et al. Promoterless gene targeting without nucleases rescues lethality of a Crigler-Najjar syndrome mouse model. *EMBO Mol. Med.* **9**, 1346–1355 (2017).
149. Borel, F. et al. Survival advantage of both human hepatocyte xenografts and genome-edited hepatocytes for treatment of alpha-1 antitrypsin deficiency. *Mol. Ther.* **25**, 2477–2489 (2017).
150. Chandler, R. J. et al. Promoterless, nuclease-free genome editing confers a growth advantage for corrected hepatocytes in mice with methylmalonic acidemia. *Hepatology* **73**, 2223–2237 (2021).
151. Cantore, A. et al. Liver-directed lentiviral gene therapy in a dog model of hemophilia B. *Sci. Transl. Med.* **7**, 277ra228 (2015).
152. Cozmescu, A. C., Counsell, J. & Gissen, P. Gene therapies targeting the liver. *J. Hepatol.* **74**, 235–236 (2021).
153. Paneda, A. et al. Safety and liver transduction efficacy of rAAV5-cohPBGD in nonhuman primates: a potential therapy for acute intermittent porphyria. *Hum. Gene Ther.* **24**, 1007–1017 (2013).
154. Gao, G. et al. Biology of AAV serotype vectors in liver-directed gene transfer to nonhuman primates. *Mol. Ther.* **13**, 77–87 (2006).
155. Greig, J. A. et al. Determining the minimally effective dose of a clinical candidate AAV vector in a mouse model of Crigler-Najjar syndrome. *Mol. Ther. Methods Clin. Dev.* **10**, 237–244 (2018).
156. Wang, L. et al. Prednisolone reduces the interferon response to AAV in cynomolgus macaques and may increase liver gene expression. *Mol. Ther. Methods Clin. Dev.* **24**, 292–305 (2022).
157. D'Avola, D. et al. Phase I open label liver-directed gene therapy clinical trial for acute intermittent porphyria. *J. Hepatol.* **65**, 776–783 (2016).
The first clinical trial of AAV for the treatment of an inherited metabolic liver disorder.
158. Baruteau, J. et al. Safety and efficacy of an engineered hepatotropic AAV gene therapy for ornithine transcarbamylase deficiency in cynomolgus monkeys. *Mol. Ther. Methods Clin. Dev.* **23**, 135–146 (2021).
159. Bell, P. et al. Inverse zonation of hepatocyte transduction with AAV vectors between mice and non-human primates. *Mol. Genet. Metab.* **104**, 395–403 (2011).
160. Ramachandran, P., Matchett, K. P., Dobie, R., Wilson-Kanamori, J. R. & Henderson, N. C. Single-cell technologies in hepatology: new insights into liver biology and disease pathogenesis. *Nat. Rev. Gastroenterol. Hepatol.* **17**, 457–472 (2020).
161. Kang, Y. B. A., Eo, J., Mert, S., Yarmush, M. L. & Usta, O. B. Metabolic patterning on a chip: towards in vitro liver zonation of primary rat and human hepatocytes. *Sci. Rep.* **8**, 8951 (2018).
162. Lisowski, L. et al. Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. *Nature* **506**, 382–386 (2014).
163. Grompe, M. & Strom, S. Mice with human livers. *Gastroenterology* **145**, 1209–1214 (2013).
164. Cabanes-Creus, M. et al. Preclinical evaluation of AAV vectors in an ex vivo human whole liver explant confirms the potential of bioengineered AAVs as clinically relevant hepatotropic vectors. *Mol. Ther.* **30**, 387 (2022).
165. George, L. A. et al. Multiyear factor VIII expression after AAV gene transfer for hemophilia A. *N. Engl. J. Med.* **385**, 1961–1973 (2021).
166. Schutgens, R. E. G. Gene therapy for hemophilia A: how long will it last? *Hemasphere* **6**, e720 (2022).
167. Takeda. *TAK-754 and TAK-748 Clinical Studies* https://www.hemophiliafed.org/uploads/Takeda-Letter-GT-Trials-Update-August-2020-HFA.pdf (2022).
168. Cheung, A. K., Hoggan, M. D., Hauswirth, W. W. & Berns, K. I. Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. *J. Virol.* **33**, 739–748 (1980).
169. Kotin, R. M. et al. Site-specific integration by adeno-associated virus. *Proc. Natl Acad. Sci. USA* **87**, 2211–2215 (1990).
170. Chiorini, J. A. et al. Biologically active Rep proteins of adeno-associated virus type 2 produced as fusion proteins in Escherichia coli. *J. Virol.* **68**, 797–804 (1994).
171. Weitzman, M. D., Kyostio, S. R., Kotin, R. M. & Owens, R. A. Adeno-associated virus (AAV) Rep proteins mediate complex formation between AAV DNA and its integration site in human DNA. *Proc. Natl Acad. Sci. USA* **91**, 5808–5812 (1994).
172. Janovitz, T. et al. High-throughput sequencing reveals principles of adeno-associated virus serotype 2 integration. *J. Virol.* **87**, 8559–8568 (2013).
173. Nault, J. C. et al. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat. Genet.* **47**, 1187–1193 (2015).
174. Smith, J. R. et al. Robust, persistent transgene expression in human embryonic stem cells is achieved with AAVS1-targeted integration. *Stem Cell* **26**, 496–504 (2008).
175. Logan, G. J. et al. Identification of liver-specific enhancer-promoter activity in the 3′ untranslated region of the wild-type AAV2 genome. *Nat. Genet.* **49**, 1267–1273 (2017).
176. La Bella, T. et al. Adeno-associated virus in the liver: natural history and consequences in tumour development. *Gut* **69**, 737–747 (2020).
177. Tatsuno, K. et al. Impact of AAV2 and hepatitis B virus integration into genome on development of hepatocellular carcinoma in patients with prior hepatitis B virus infection. *Clin. Cancer Res.* **25**, 6217–6227 (2019).
178. Park, K. J. et al. Adeno-associated virus 2-mediated hepatocellular carcinoma is very rare in Korean patients. *Ann. Lab. Med.* **36**, 469–474 (2016).
179. Nault, J. C. et al. Wild-type AAV insertions in hepatocellular carcinoma do not inform debate over genotoxicity risk of vectorized AAV. *Mol. Ther.* **24**, 660–661 (2016).
180. Schmidt, M., Gil-Farina, I. & Buning, H. Reply to "Wild-type AAV insertions in hepatocellular carcinoma do not inform debate over genotoxicity risk of vectorized AAV". *Mol. Ther.* **24**, 661–662 (2016).
181. Schaffer, A. A. et al. Integration of adeno-associated virus (AAV) into the genomes of most Thai and Mongolian liver cancer patients does not induce oncogenesis. *BMC Genomics* **22**, 814 (2021).
182. Bayard, Q. et al. Cyclin A2/E1 activation defines a hepatocellular carcinoma subclass with a rearrangement signature of replication stress. *Nat. Commun.* **9**, 5235 (2018).
183. Cantalupo, P. G., Katz, J. P. & Pipas, J. M. Viral sequences in human cancer. *Virology* **513**, 208–216 (2018).
184. Zapatka, M. et al. The landscape of viral associations in human cancers. *Nat. Genet.* **52**, 320–330 (2020).
185. Srivastava, A. & Carter, B. J. AAV infection: protection from cancer. *Hum. Gene Ther.* **28**, 323–327 (2017).
186. Ostrove, J. M., Duckworth, D. H. & Berns, K. I. Inhibition of adenovirus-transformed cell oncogenicity by adeno-associated virus. *Virology* **113**, 521–533 (1981).
187. Raj, K., Ogston, P. & Beard, P. Virus-mediated killing of cells that lack p53 activity. *Nature* **412**, 914–917 (2001).
188. Mayor, H. D., Drake, S., Stahmann, J. & Mumford, D. M. Antibodies to adeno-associated satellite virus and herpes simplex in sera from cancer patients and normal adults. *Am. J. Obstet. Gynecol.* **126**, 100–104 (1976).
189. Sabatino, D. E. et al. Evaluating the state of the science for adeno-associated virus (AAV) integration: an integrated perspective. *Mol. Ther.* https://doi.org/10.1016/j.ymthe.2022.06.004 (2022).
This article provides the perspectives of leading experts in the field of AAV integration about the safety of AAVs and their potential genotoxicity.
190. Rutledge, E. A. & Russell, D. W. Adeno-associated virus vector integration junctions. *J. Virol.* **71**, 8429–8436 (1997).
191. Miller, D. G., Petek, L. M. & Russell, D. W. Adeno-associated virus vectors integrate at chromosome breakage sites. *Nat. Genet.* **36**, 767–773 (2004).
In this study, an elegant system was developed for testing the hypothesis that AAV vectors integrate at existing DNA double-strand breaks without causing breaks themselves.
192. Philpott, N. J., Gomos, J. & Falck-Pedersen, E. Transgene expression after rep-mediated site-specific integration into chromosome 19. *Hum. Gene Ther.* **15**, 47–61 (2004).
193. Chen, W. Y. & Townes, T. M. Molecular mechanism for silencing virally transduced genes involves histone deacetylation and chromatin condensation. *Proc. Natl Acad. Sci. USA* **97**, 377–382 (2000).
194. Nakai, H. et al. Extrachromosomal recombinant adeno-associated virus vector genomes are primarily responsible for stable liver transduction in vivo. *J. Virol.* **75**, 6969–6976 (2001).
195. Duan, D. et al. Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J. Virol.* **72**, 8568–8577 (1998).
This study showed the episomal nature of recombinant AAV genomes responsible for long-term transgene expression.
196. Nakai, H. et al. AAV serotype 2 vectors preferentially integrate into active genes in mice. *Nat. Genet.* **34**, 297–302 (2003).
The first study to establish the techniques to characterize the integration pattern of AAV vectors.
197. Nakai, H. et al. Large-scale molecular characterization of adeno-associated virus vector integration in mouse liver. *J. Virol.* **79**, 3606–3614 (2005).
198. Inagaki, K. et al. DNA palindromes with a modest arm length of greater, similar 20 base pairs are a significant target for recombinant adeno-associated virus vector integration in the liver, muscles, and heart in mice. *J. Virol.* **81**, 11290–11303 (2007).
199. Donsante, A. et al. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene Ther.* **8**, 1343–1346 (2001).

200. Bell, P. et al. Analysis of tumors arising in male B6C3F1 mice with and without AAV vector delivery to liver. *Mol. Ther.* **14**, 34–44 (2006).
201. Donsante, A. et al. AAV vector integration sites in mouse hepatocellular carcinoma. *Science* **317**, 477 (2007).
202. Chandler, R. J. et al. Vector design influences hepatic genotoxicity after adeno-associated virus gene therapy. *J. Clin. Invest.* **125**, 870–880 (2015).
The most comprehensive preclinical study of the risk of genotoxicity with liver-directed AAV therapies, in which some of the important factors influencing genotoxicity are defined.
203. Dalwadi, D. A. et al. Liver injury increases the incidence of HCC following AAV gene therapy in mice. *Mol. Ther.* **29**, 680–690 (2021).
204. Dalwadi, D. A. et al. AAV integration in human hepatocytes. *Mol. Ther.* **29**, 2898–2909 (2021).
205. Inagaki, K., Piao, C., Kotchey, N. M., Wu, X. & Nakai, H. Frequency and spectrum of genomic integration of recombinant adeno-associated virus serotype 8 vector in neonatal mouse liver. *J. Virol.* **82**, 9513–9524 (2008).
206. Zhong, L. et al. Recombinant adeno-associated virus integration sites in murine liver after ornithine transcarbamylase gene correction. *Hum. Gene Ther.* **24**, 520–525 (2013).
207. Rosas, L. E. et al. Patterns of scAAV vector insertion associated with oncogenic events in a mouse model for genotoxicity. *Mol. Ther.* **20**, 2098–2110 (2012).
208. Bell, P. et al. No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. *Mol. Ther.* **12**, 299–306 (2005).
209. Li, H. et al. Assessing the potential for AAV vector genotoxicity in a murine model. *Blood* **117**, 3311–3319 (2011).
210. BioMarin. U.S. *FDA Placed a Clinical Hold on BMN 307 Phearless Phase ½ Gene Therapy Study in Adults with PKU Based on Interim Pre-clinical Study Findings* https://investors.biomarin.com/2021-09-06-U-S-FDA-Placed-a-Clinical-Hold-on-BMN-307-Phearless-Phase-1-2-Gene-Therapy-Study-in-Adults-with-PKU-Based-on-Interim-Pre-clinical-Study-Findings (2021).
211. Nguyen, G. N. et al. A long-term study of AAV gene therapy in dogs with hemophilia A identifies clonal expansions of transduced liver cells. *Nat. Biotechnol.* **39**, 47–55 (2021).
A 10-year follow-up of AAV gene therapy in dogs that provided long-term safety and expression data and a thorough analysis of vector integration characteristics.
212. Nowrouzi, A. et al. Integration frequency and intermolecular recombination of rAAV vectors in non-human primate skeletal muscle and liver. *Mol. Ther.* **20**, 1177–1186 (2012).
213. Gil-Farina, I. et al. Recombinant AAV integration is not associated with hepatic genotoxicity in nonhuman primates and patients. *Mol. Ther.* **24**, 1100–1105 (2016).
214. Kaeppel, C. et al. A largely random AAV integration profile after LPLD gene therapy. *Nat. Med.* **19**, 889–891 (2013).
215. Euroland. uniQure Announces Clinical Update on Hemophilia B Gene Therapy Program. *uniQure* https://uniqure.gcs-web.com/node/10231/pdf (2021).
216. Hordeaux, J. et al. Safe and sustained expression of human iduronidase after intrathecal administration of adeno-associated virus serotype 9 in infant Rhesus monkeys. *Hum. Gene Ther.* **30**, 957–966 (2019).
217. Kang, H. R. et al. Pathogenesis of hepatic tumors following gene therapy in murine and canine models of glycogen storage disease. *Mol. Ther. Methods Clin. Dev.* **15**, 383–391 (2019).
218. Chen, S., Yao, Y., Zhang, Y. & Fan, G. CRISPR system: discovery, development and off-target detection. *Cell Signal.* **70**, 109577 (2020).
219. Musunuru, K. et al. In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates. *Nature* **593**, 429–434 (2021).
220. Rothgangl, T. et al. In vivo adenine base editing of PCSK9 in macaques reduces LDL cholesterol levels. *Nat. Biotechnol.* **39**, 949–957 (2021).
221. Muller, H. P. & Schaffner, W. Transcriptional enhancers can act in trans. *Trends Genet.* **6**, 300–304 (1990).
222. Kadauke, S. & Blobel, G. A. Chromatin loops in gene regulation. *Biochim. Biophys. Acta* **1789**, 17–25 (2009).
223. Schmidt, F., Kern, F. & Schulz, M. H. Integrative prediction of gene expression with chromatin accessibility and conformation data. *Epigenetics Chromatin* **13**, 4 (2020).
224. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT00076557 (2004).
225. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT00979238 (2009).
226. Nathwani, A. C. et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N. Engl. J. Med.* **371**, 1994–2004 (2014).
227. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT02396342 (2015).
228. Miesbach, W. et al. Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B. *Blood* **131**, 1022–1031 (2018).
229. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT01687608 (2012).
230. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03489291 (2018).
231. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03369444 (2017).
232. Chowdary, P. et al. Phase 1-2 trial of AAVS3 gene therapy in patients with hemophilia B. *N. Engl. J. Med.* **387**, 237–247 (2022).
233. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT02618915 (2018).
234. Pipe, S. in AHS Annual Meeting & Exposition Vol. 130, 3331 (AHS, 2017).
235. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT02576795 (2015).
236. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03003533 (2016).
237. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03061201 (2022).
238. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03588299 (2018).
239. Pipe, S. W. in ASH Annual Meeting & Exposition Vol. 136, 44–45 (2020).
240. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT03370172 (2017).
241. GlobeNewswire. *Ultragenyx Completes Successful End-of-Phase 2 Meeting with FDA and Finalizes Phase 3 Study Design for DTX301 Ornithine Transcarbamylase (OTC) Gene Therapy Program* https://www.globenewswire.com/news-release/2021/04/22/2215092/20739/en/Ultragenyx-Completes-Successful-End-of-Phase-2-Meeting-with-FDA-and-Finalizes-Phase-3-Study-Design-for-DTX301-Ornithine-Transcarbamylase-OTC-Gene-Therapy-Program.html (2021).
242. GlobeNewswire. *Homology Medicines Announces Presentation of Positive Data from the Dose-Escalation Phase of the pheNIX Gene Therapy Trial for Adults with PKU* https://www.globenewswire.com/news-release/2020/11/06/2122133/0/en/Homology-Medicines-Announces-Presentation-of-Positive-Data-from-the-Dose-Escalation-Phase-of-the-pheNIX-Gene-Therapy-Trial-for-Adults-with-PKU.html (2020).
243. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT04480567 (2020).
244. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT04452513 (2020).
245. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT05222178 (2022).
246. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT02082860 (2014).
247. PR Newswire. *LogicBio Therapeutics Announces FDA Lifts Clinical Hold on SUNRISE Trial in Pediatric Patients with Methylmalonic Acidemia* https://www.prnewswire.com/news-releases/logicbio-therapeutics-announces-fda-lifts-clinical-hold-on-sunrise-trial-in-pediatric-patients-with-methylmalonic-acidemia-301542452.html (2022).
248. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT02651675 (2016).
249. Ultragenix. *Ultragenix announces positive data from Phase 1/2 study of DTX401 gene therapy in Glycogen Storage Disease type Ia* https://ir.ultragenyx.com/news-releases/news-release-details/ultragenyx-announces-positive-data-phase-12-study-dtx401-gene (2019).
250. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT04537377 (2020).
251. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT04884815 (2021).
252. US National Library of Medicine. *ClinicalTrials.gov* https://ClinicalTrials.gov/show/NCT04601051 (2020).
253. Bonnemann, C. G. A Collaborative Analysis by Clinical Trial Sponsors and Academic Experts of Anti-transgene SAEs in Studies of Gene Therapy for DMD in 25th Annual Meeting of the American Society of Gene & Cell Therapy. *Mol. Ther.* **30**, S1–S592 (2022).

Author contributions

N.Z., C.U. and G.G.-A. researched data for the article. All authors made substantia contributions to the discussion of content, contributed to writing, and reviewed and edited the manuscript before submission.

Competing interests

N.D.W. and G.G-A. are employees and shareholders of Vivet Therapeutics. The views expressed in this Review belong to the authors alone and do not reflect the opinions of Vivet Therapeutics. The other authors declare no competing interests.

Additional information

Correspondence should be addressed to Gloria Gonzalez-Aseguinolaza.

Peer review information *Nature Reviews Gastroenterology & Hepatology* thanks M. Grompe, G. Ronzitti and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.