Review

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Adeno-associated viruses for gene therapy – clinical implications and liver-related complications, a guide for hepatologists

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Summary

Gene therapy has garnered increasing interest over recent decades. Several therapies employing gene transfer mechanisms have been developed, and, of these, adeno-associated virus (AAV) vectors have demonstrated viability for use with *in vivo* gene therapy. Several AAV-based therapeutics have received regulatory approval in the last few years including those for retinal disease, spinal muscular atrophy or aromatic L-amino acid decarboxylase deficiency. Lately, with the introduction of novel liver-directed AAV vector-based therapeutics for the treatment of haemophilia A and B, gene therapy has attracted significant attention in the hepatology community, with the liver increasingly recognised as a target for gene therapy. However, the introduction of foreign DNA into hepatocytes is associated with a risk of hepatic reactions, with raised ALT (alanine aminotransferase) and AST (aspartate aminotransferase) being – so far – the most commonly reported side effects. The complete mechanisms underlying the ALT flairs remain to be determined and the long-term risks associated with these new treatments is not yet known. The liver community is increasingly being asked to support liver-directed gene therapy to mitigate potential liver associated harm. In this review, we focus on AAV vector-based gene therapy, shedding light on this promising technique and its remarkable success in haemophilia, with a special focus on hepatic complications and their management in daily clinical practice.

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Introduction

Gene therapy – the introduction of genetic material into patients to modify gene or protein expression for therapeutic benefit – has become a field of growing interest over the last decades. In the field of hepatology, recent advances in gene silencing with small-interfering RNA (siRNA) have led to novel therapeutic options, such as givosiran for the treatment of acute hepatic porphyria or fazirsiran to treat patients with alpha$_1$-antitrypsin deficiency.[1,2] Other siRNAs are currently being evaluated for the treatment and cure of patients with chronic hepatitis B virus infection[3] (Fig. 1).

Until recently, gene therapy itself, involving the transfection of desired gene(s) using different viral or bacterial vectors, has not played an important role for hepatologists, but this picture is now changing. Over the past decade, several therapies employing gene transfer have become available and, adeno-associated virus (AAV) vectors have proved popular mechanistically and are increasingly being used for *in vivo* gene therapy. Both the European Medicines Agency (EMA) and US Food and Drug Administration (FDA) have approved several AAV-based therapeutics in the last few years, including those for retinal disease, spinal muscular atrophy or aromatic L-amino acid decarboxylase deficiency.[4,5] Others are being fast-tracked for clinical approval, *e.g.* congestive heart failure, x-lined myotubular myopathy, glioma or glioblastoma.[6] An overview of all (conditionally) approved AAV-based gene therapies is depicted in Table 1. The introduction of several novel liver-directed AAV vector-based therapeutics for the treatment of haemophilia A and B has led to gene therapy attracting significant attention in the hepatology community, with the liver increasingly seen as a target for gene therapy. However, insertion of foreign DNA into hepatocytes to induce transgene expression may result in hepatic reactions. Commonly reported side effects include drug-related hepatitis which can be diagnosed through increased serum alanine aminotransferase (ALT) that may also be accompanied by reduced transgene expression. Elevated liver enzymes were reported in earlier trials involving intravenous injections of a non-liver-directed AAV-based gene therapy for spinal muscular atrophy, possibly related to immunogenicity acquired through past AAV infections.[7,8] A recent single case report documented the occurrence of hepatocellular carcinoma in a patient receiving liver-directed AAV5 therapy for haemophilia B in the HOPE-B clinical trial, though this patient had several pre-existing oncogenic risk factors that were believed to be the most

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Keypoints

- Adeno-associated virus (AAV)-mediated gene therapy is increasingly being used to manage genetic disorders and gene therapy treatments for haemophilia are now licensed in Europe.
- A common side-effect of AAV-mediated gene therapy is a transient transaminitis that is not associated with significant liver damage nor changes in liver function (albumin and bilirubin).
- The aetiology of the AAV-associated hepatitis is uncertain and both innate and acquired immune responses have been implicated.
- In patients with haemophilia, the AAV-associated hepatitis may be linked to a reduction in the efficacy of gene therapy – *i.e.* expression of the transgene diminishes and, in some patients, expression can be restored by the use of prednisolone and other immunosuppressants.
- There is an emerging consensus that AAV-associated hepatitis should be promptly treated with immunosuppression in a similar manner to auto-immune hepatitis but this therapy should not be continued if it does not lead to restoration of transgene expression.
- Long-term (over several years) reduction in the expression of AAV-introduced transgenes seems to be associated with reductions in transcription of the introduced DNA rather than transgene loss and may therefore be reversible, although this has not yet been demonstrated.
- Hepatologists will need to liase closely with their haemophilia colleagues to ensure that the benefits of these new therapeutics can be realised.

likely cause of the malignancy.[9] Hence the liver community is increasingly being asked to support gene therapy programmes to mitigate potential liver associated harm. In this report, we focus on AAV vector-based gene therapy, shedding light on this promising technique and its remarkable success in haemophilia, with a special focus on potential hepatic complications and their management in daily clinical practice.

Virology and mechanism of AAV transfection

AAVs are 20 nanometer, icosahedral structures of the Parvoviridae family that were first discovered as a contaminant in adenovirus preparations.[10] They are non-enveloped DNA viruses that contain a linear single-stranded genome of about 4.7 kb encoding for viral replication, packaging and capsid assembly, flanked by two inverted terminal repeats (ITRs).[10] There are at least 11 different AAV subtypes which have subtly different tissue tropisms due to differing interactions with their receptor.[11]

The first description of recombinant AAVs dates back to 1989.[12] Since then, AAV-based vectors have been engineered through the replacement of the viral genes of wild-type AAVs with a synthetic gene expression cassette containing transcriptional regulatory elements (enhancer/promoter sequences),

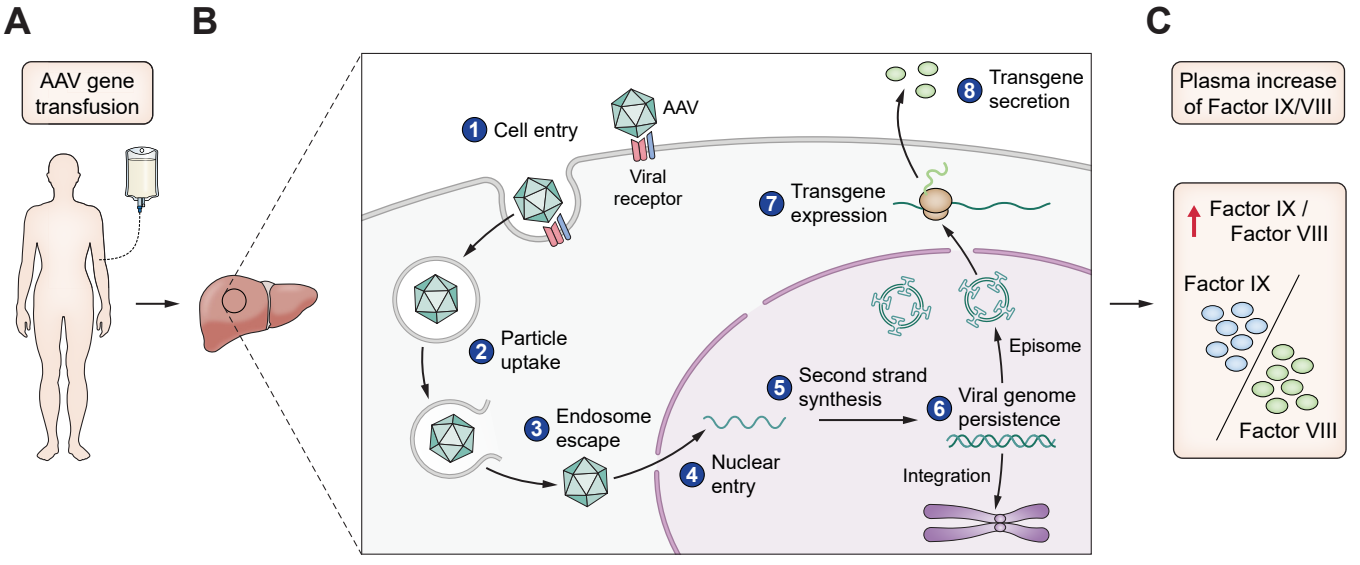

Fig. 1. Liver-directed AAV gene therapy for haemophilia. (A) AAV gene transfusion, (B) vector transduction and intracellular life cycle: 1) entry into the cell requires binding to a cell type-specific receptor and often a co-receptor to enhance transduction efficiency; 2) uptake of the vector particle into an endosome and trafficking through the cytoplasm; 3) endosome escape of the vector genome and subsequent capsid proteolysis; 4) entry of the single stranded vector genome into the nucleus; 5) synthesis of second strand of vector DNA; 6) vector genomes persistent as either concatemeric circular episomes (significant majority of vector), or as integrated vector copies [significant minority of the vector often rearranged and truncated]; 7) transgene mRNA expression; 8) transgene protein translation and secretion) resulting in (C) a plasma increase of factor VIII/IX.

Table 1. Currently (conditional) approved AAV-based gene therapies (by FDA or EMA) and reported hepatic adverse events in phase III clinical trials.

Therapeutic substance	Approval for treatment	Vector	Administration route	Hepatic adverse events*	Note*
Voretigene neparvovec	Retinal disease	AAV2-based	Subretinal injection	None[84]	Prophylactic prednisolone (1 mg/kg) over 7 days
Onasemnogene abeparvovec	Spinal muscular atrophy	AAV9-based	Intravenous infusion	Elevation of transaminases (n = 7/22,31.8%), one SAE[7]	Prophylactic prednisolone (1 mg/kg) tapered over 30 days, and for ALT elevations
Ladocagene exuparvovec	Aromatic L-amino acid decarboxylase deficiency	AAV2	Putamen injection	None[85]	No prednisolone
Valoctocogene roxaparvovec	Haemophilia A	AAV5	Intravenous infusion	Elevation of transaminases (n = 115/134)[37]	Prednisolone per protocol for ALT elevations
Etranacogene dezaparvovec	Haemophilia B	AAV5	Intravenous infusion	Elevation of transaminases (n = 11/54)	Prednisolone per protocol for ALT elevations

AAV, adeno-associated virus; ALT, alanine aminotransferase; SAE, serious adverse event.
*Reported in phase III trials.

the transgene of interest and a transcriptional termination element (*e.g.* polyadenylation sequence). The ITRs are the only remaining wild-type viral sequences and they function to mediate vector genome packaging and to enhance stability of the vector genome – notably, they are retained in therapeutic vectors.[10] Recombinant AAVs are unable to replicate *in vivo* which is believed to reduce their capacity to mutate, which may render them safe vehicles for long-term transgene expression following a single intravenous administration.

AAV vectors can be manufactured in mammalian cells (*e.g.* HeLa cells or human embryonic kidney [HEK] 293 cells) or insect cell systems (baculovirus-*Spodoptera frugiperda* [Sf]. The production of AAV vectors in insect cell systems results in high vector concentrations, as is required for clinical administration into humans. However, these vector structures tend to be more heterogenous with more truncated and unresolved genomes compared to those produced in mammalian cells.[13] Despite this, the long-term durability of AAV5 vectors produced by both manufacturing systems is similar.[14]

Following vector infusion, rapid transduction occurs via receptor-mediated uptake into endosomes. Importantly, distinct structures on the AAV vector capsid mediate binding via primary receptors (*e.g.* glycans and proteoglycans) that are distinct for different tissues. Vector particle internalisation is further facilitated by interactions with secondary membrane protein receptors. As a result, differing capsid configurations among AAV serotypes lead to specific tissue tropisms (many primarily targeting the liver) that can be used or modified in clinical settings to guide transgene therapy to the desired target cells.[15] Following cellular uptake, intracellular trafficking occurs in endosomes before endosomal escape of the vector genome, and the process of capsid degradation. The vector genome then enters the nucleus where a second DNA strand is synthesised and the majority (>99%) of these double-stranded sequences form monomeric and concatemeric circular extrachromosomal structures (episomes).[10] However, despite lacking integrase capabilities, vector sequences (usually truncated or rearranged) may integrate into the host cell genome at sites of DNA damage and fragility. Although not fully resolved, there is evidence suggesting the majority of long-term transgene expression derives from episomal vector copies. Different factors impacting the efficacy of AAV transduction have been described: *e.g.* vector uptake, endosomal escape, capsid uncoating of single-stranded DNA in the nucleous.[16] Additionally, multiple processes involving the host machinery are involved in targeting, expression and secretion of the therapeutic protein. Hence, individual expression of host factors may influence the level of response and result in significant inter-individual variability of transgene expression, observed in preclinical and clinical studies.[17,18]

While early gene therapy vectors focused on integrating viruses and were associated with rare cases of oncogenesis, recombinant AAVs do not integrate into the host genome through some active process.[10] This potentially reduces or avoids certain oncogenic effects that have been, conversely, documented with retroviral vectors, *i.e.* insertional mutagenesis or the enhanced expression of unrelated genes located close to the vector insertion site. However, a small percentage of AAV vector integration (~1 in 1,000 cells) does occur and thus, as vector doses in adult patients can involve the infusion of $>10^{15}$ vector particles, this will result in millions of integrated sequences in hepatocytes.[19,20] Despite this, there is no clear evidence of a pathogenic role for AAV in malignant tumour generation in the large number of patients treated with AAV gene therapy to date (see below).[21]

Until recently, AAV was not thought to be pathogenic in humans. The recent description of an unusual, severe hepatitis in children thought to be due to an atypical immune response to AAV infection challenges this assumption.[22,23] It is unclear whether this rare response is possible in adult patients nor is it clear whether the therapeutic vectors are capable of such a reaction. To-date no cases of severe dysfunction have been reported in people with haemophilia receiving gene therapy.

Although AAV vectors are significantly less immunogenic than adenoviral vectors, immunogenicity remains an important issue as it can interfere with sustained AAV expression. Many healthy individuals have been in contact with different wild-type AAVs and these prior exposures can elicit neutralising antibodies that can effectively block AAV gene delivery or lead to the destruction of transduced cells by $CD8^+$ cytotoxic T lymphocytes.[24] Interestingly, this phenomenon was first described in humans and has not been observed in prior (wild-type AAV-naïve) animal models. Following recombinant AAV infusion, the vector capsid induces a strong humoral immune response leading to the production of high-titer anti-AAV-neutralising antibodies that persist for many years and thus likely prevent re-administration of AAV-mediated gene therapy.[25–27] Hence, at present, AAV-mediated gene therapy is a 'one-shot' therapy, which is a significant stimulus to develop approaches to maximise its success – loss of transgene expression is currently understood to lead to loss of the therapeutic effect. Whether new

and more efficacious immunosuppressive regimens that will enable repeat dosing will be developed in the future remains to be seen but, at present, gene therapy with AAV vectors should be regarded as unrepeatable.

Liver-directed AAV gene therapy in patients with haemophilia

Most recently, AAV gene therapy has attracted attention for its success in treating patients with haemophilia – with two therapeutics already approved by the EMA and the FDA (Table 1). As these therapeutics provide a first glance at liver-directed AAV gene therapy in a larger patient population in a clinical setting, they will be discussed here in more detail.

Haemophilia is an X-chromosomal recessive, hereditary bleeding disorder resulting in a deficiency of coagulation factor VIII (FVIII – haemophilia A) or FIX (haemophilia B).[28] About 50% of patients suffer from moderate to severe haemophilia and those with severe hemophilia require intravenous administration of factor concentrate several times per week to prevent spontaneous bleeding (especially joint bleeds) and resulting arthropathy.[29] With the development of long-acting FVIII and FIX products, infusion intervals can be extended; subcutaneous treatment with emicizumab, a FVIII mimetic antibody, can extend treatment intervals further for those with haemophilia A.[30] Yet, continued bleeding into the joint cavity means that many patients still develop arthropathy over time despite access to these treatments.[31]

Gene therapy for haemophilia is especially attractive as it is a monogenetic disease. Moreover, a minimal increase in FVIII or FIX levels results in a major reduction in bleeding events (severe haemophilia <1 IU/dl, moderate haemophilia 1-5 IU/dl), with plasma levels of 5-40 IU/dl already considered mild haemophilia with low risk of bleeding events – an increase to this level would usually be regarded as clinically relevant. Therapeutic efficacy can be readily monitored by easily available blood tests. The genetic transcript is small and can be packaged in an AAV, and gene expression can be easily evaluated by measuring factor levels in plasma.[28,32]

In 2006, a AAV2-based liver-directed gene therapy was administered via the hepatic artery in the first clinical study in patients with haemophilia B. FIX expression was significantly increased, however only a short-term response could be achieved.[33] Five years later an AAV8-based intravenously administered gene therapy resulted in the long-lasting AAV-mediated expression of FIX at 2 to 11% of normal levels.[24] Consequently, a significant reduction of bleeding events was observed without the need for prophylaxis.[34,35]

These trials have paved the way for at least 65 studies investigating gene therapy in people with haemophilia.[36] However, as most studies are phase I or II, include limited numbers of patients and use non-randomised designs, data are still limited. Currently, the results of two phase III trials – one each for haemophilia A and B –have been published, resulting in (conditional) approval of both drugs for haemophilia treatment:

GENEr8-1 is an open-label, single-group, multicenter study in adult men with severe haemophilia A (FVIII ≤1 IU/dl) without preexisting anti-AAV5 antibodies or history of factor VIII inhibitors who were receiving routine FVIII prophylaxis.[37] Patients received a single infusion of valoctocogene roxaparvovec (AAV5-hFVIII-SQ, $6x10^{13}$ vector genomes/kg) and were followed for 52 weeks. The primary endpoint was the change from baseline in factor VIII activity during weeks 49 through 52 after infusion. Overall, 134 patients received an infusion, 132 were human immunodeficiency virus negative. Factor VIII activity increased by 41.9 IU/dl (p <0.001) and treated bleeding events decreased by 83.8% (p <0.001). All patients had at least one adverse event (AE), 22 had serious AEs (SAEs). Elevations in ALT levels were reported in 115 (85.8%) patients and were treated with immunosuppressive agents, mainly glucocorticoids, with 96.2% of events resolved by data cut-off. ALT elevations were deemed severe in two patients who required intravenous methylprednisolone. Based on the data published in this trial, valoctocogene roxaparvovec was approved in the European Union in 2022 and its application to the US FDA is under review.[38] Recently an update was published analysing safety and efficacy 2 years following gene therapy: valoctocogene roxaparvovec showed a durable increase in factor VIII activity and a reduction in bleeding, while no new treatment-related SAEs or safety signals emerged.[39]

HOPE-B is an open-label, single-dose, single-arm, multinational trial in adult males with severe or moderate-severe haemophilia B (FIX ≤2%) who received routine FIX prophylaxis prior to the study.[40] After an observational period of at least 6 months during which bleeding/factor use was monitored, patients received a single intravenous dose of etranacogene dezaparvovec (AAV5-PdFIX, $2x10^{13}$ gc/kg) and were followed for an additional 5 years. The primary endpoints were FIX activity at 26 and 52 weeks after dosing and 52-week annualised bleeding rate. Overall, 54 patients were dosed. FIX activity increased rapidly after gene therapy from baseline to a least-squares mean of 36.2% at 6 months and 34.3% at 18 months (both p <0.001). No correlation of pre-existing neutralising antibodies was identified up to a titre of 678, yet a patient with a titre of 3,212 did not respond. The 52-week adjusted annualised bleeding rate was reduced by 64% (p = 0.0002) when compared to FIX prophylaxis. FIX-treated bleeds were reduced by 77% (p <0.0001). No treatment-related SAEs were reported, 37 patients reported treatment-related AEs, 9 patients with elevation in liver enzymes who received steroids per protocol. All patients discontinued steroid use prior to week 26 and FIX activity was preserved in the mild range. Data on these trials have resulted in the (conditional) approval of etranacogene dezaparvovec for the treatment of haemophilia B in the US and Europe.[4,5]

Possible mechanisms causing ALT elevation after AAV gene therapy

As the liver is one of the body's principal organs involved in the essential synthesis of many proteins and biochemicals, it has become an important organ for transgene expression. Studies in large animals and humans have demonstrated that liver-directed AAV vector delivery is feasible and can produce multi-year transgene expression after a single intravenous transfer of the missing or defective gene.[41] However, liver safety is a pivotal consideration. In fact, AAV's tropism for the liver makes it an attractive organ for all AAV-mediated gene therapies, leading to a relatively high number of genomes successfully delivered to hepatocytes. However, a typically symptomatic inflammatory response may be observed in the majority of recipients. Lately, clinical studies involving people

with haemophilia have demonstrated the usefulness of AAV-directed gene therapy as a powerful tool in clinical settings; yet elevated liver enzymes in the months following vector administration can be detected in the majority of patients through regular clinical assessment.[37,40]

The precise mechanisms of post-AAV transient transaminitis are not completely understood. It is noteworthy that there is less liver inflammation in patients undergoing therapy for haemophilia B (with FIX being expressed in its normal site of origin, the hepatocyte) than for haemophilia A (where FVIII that is normally produced in sinusoidal cells is generated in a different cell, the hepatocyte),[42] perhaps suggesting that ectopic expression of the protein may enhance any inflammatory response. It is also possible FVIII protein is inherently more difficult to fold and secrete than FIX protein.[43] Studies on other gene therapies that are currently being tested in clinical trials (See Table 1) have also reported hepatic side effects, but few mechanistic details or insights are currently available. Liver biopsies obtained from gene therapy recipients at the time of ALT increase may be necessary to further unravel the mechanisms involved, but it is not yet clear when these should be performed. Clinical studies with protocol biopsies at pre-specified times following the onset of the associated transaminitis would be of great help in advancing our understanding of the underlying mechanisms. At present, a number of putative mechanisms have been proposed, including immune-mediated and cellular stress-induced damage to hepatocytes, which have both been hypothesised to cause transaminase elevation and can result in reduced transgene expression.

As AAV vectors have only a limited capacity (~4.7 kb), a shorter BDD-FVIII transgene is used for haemophilia A gene therapy. However, the BDD-FVIII protein is prone to misfolding and may accumulate in the endoplasmic reticulum (ER). This may be problematic as the per cell content of FVIII DNA cannot be precisely controlled and an elegant hypothesis to explain the increased incidence of ALT elevations in people treated with FVIII rather than FIX gene therapies is that intracellular stress is an important contributing factor. In fact, over-expression of BDD-FVIII in hepatocytes has been shown to induce an unfolded protein response, leading to ER stress and eventual apoptosis.[44–46] Several groups also reported such an unfolded protein response as a result of AAV-BDD FVIII gene therapy in animal models.[18,47–49] Fong *et al.* reported that a more potent promoter resulted in very high expression of FVIII per cell and an ER stress response was detected in rodents receiving the highest dose and strongest promotor.[18] Others saw, in different experimental settings, an unfolded protein response and evidence of translational shut down in some mice, yet this was not correlated with high FVIII expression levels. Importantly, increased cellular stress was not associated with the elevation of liver enzymes in any of these preclinical studies, which rather undermines the hypothesis that cellular stress is the dominant causative factor of ALT increases. However, the number of humans studied in this study was very small and, at this stage, it is not possible to exclude the possibility that an unfolded protein response plays a role in AAV-associated transaminitis.

Another mechanism triggering ALT elevation in these patients is hypothesised to be a capsid-mediated immune response. The first evidence of such an immune response following liver gene transfer was observed in patients with haemophilia B who received AAV2-FIX via the hepatic artery.[24] In these patients, FIX expression was particularly short-lived (longer lasting therapeutic efficacy was expected from studies in haemophilic dogs) and transient elevated liver enzymes were reported. The elevation in transaminases was linked to the development of $CD8^+$ T-cell responses to specific sequences in the AAV capsid.[24,50,51] AAV-transduced hepatocytes that present AAV capsid antigens loaded on MHC-I complexes may then be targeted and eliminated by these cytotoxic T cells. In another clinical study, in two out of six patients with severe haemophilia B receiving intravenous AAV8-FIX without immunosuppression, increased ALT values were reported in those who received the highest vector dose. Both patients were started on steroid therapy and transaminases normalised thereafter while FIX levels were maintained.[33] At present, immune-mediated damage to transduced hepatocytes is thought to play a significant role in the post-treatment hepatitis seen in many patients undergoing treatment. Since the hepatitis may be associated with a reduction in factor expression that is sometimes reversed by immunosuppressive therapy, clinical practice and licensing guidelines support the use of immunosuppressive therapies in patients who develop liver injury. However, it is important to note that this approach has never been subjected to a randomised trial.

Since the early reports of benefits of immunosuppression in patients who develop post-treatment hepatitis, strategies have been developed to limit vector dose and immunosuppressive protocols have been defined in cases where ALT elevation occurred in subsequent clinical trials.[51] Although corticosteroids constitute the backbone of such protocols, responses are mixed and the exact mechanisms leading to ALT elevations are incompletely understood. So far, preclinical models (mouse or non-human primates) have proven imprecise in predicting immunogenicity and subsequent hepatitis. Moreover, in both clinical trials for the treatment of spinal muscular atrophy in which an AAV9 vector was used, similar ALT elevations were reported,[7,8] which may relate to the high liver tropism of this vector.

A number of preclinical studies have also linked the innate immune system with an important role in this scenario.[52–54] The unmethylated dinucleotide CpG motifs in DNA can act as pathogen-associated molecular patterns that initiate immune responses by activating Toll-like receptor 9. In the creation of gene therapy vectors, these potentially pathogenic coding sequences are usually minimised by different codon-modification strategies, as studies have shown that these sequences may be a key factor that initiates damaging responses leading to inflammation and reduced expression.[55] Recently, a clinical trial revealed an important role for Toll-like receptor 9 activation as a trigger for anti-capsid cytotoxic T-cell responses in patients with haemophilia. In this trial, long-term transgene expression failed in all but one patient. This patient had a functional polymorphism in the gene for the IL-6 receptor which is associated with the disruption of regular pro-inflammatory signaling.[56]

The details of the interactions between the innate and acquired immune responses that lead to gene therapy-associated transaminitis remain to be elucidated but it is likely that both contribute to some extent and the impact of each is likely to be dependent, to some extent, on the host. It is important to note that AAV can infect organs other than the liver. This is probably of minor importance in the treatment of haemophilia but in other therapeutic areas very much higher amounts of virus are infused and there have been reports of damage to other organs

(chiefly the kidney and heart) with one recent report of a fatal response to treatment for Duchenne muscular dystrophy.[57] The doses of AAV used to treat muscular dystrophy are greater than those used in haemophilia (1x 10^{14} vg/kg in muscular dystrophy cf 2x10^{13} gc/kg in the HOPE-B trial) and the vector in the recent fatal case was of a different serotype, but treating physicians should be aware of the potential for other organ involvement and, as we increasingly deploy these transformative new therapies, continued vigilance will be needed.

Clinical presentation and management of ALT elevations

The best available data on ALT elevations in larger patient cohorts receiving liver-directed AAV gene therapy is derived from the two phase III clinical trials for haemophilia involving valoctocogene roxaparvovec (GENEr8-1 trial) and etranacogene dezaparvovec (HOPE-B trial).[32,37]

In the GENEr8-1 trial, most patients developed ALT elevations (85.8%) during treatment.[37] Details on ALT elevation and glucocorticoid use are depicted in Table 2. ALT elevations occurred a median of 8 weeks after AAV-infusion. However, early onset ALT elevations have also been described after 1 week. ALT increments were mild in most cases (61.9% Grade 1), but moderate to severe in 15.7% and 8.2% of cases, respectively. The majority of patients received glucocorticoid treatment – about 80% were treated with oral prednisone or prednisolone at a dose of 60 mg per day which was tapered over a period of at least 8 weeks. Of note, as ALT events often occurred more than once (75 patients with >1 event), the median duration of glucocorticoid therapy per participant was 230 days in this trial and AEs related to glucocorticoid therapy were common (72% of patients). The most common AEs included – among others – acne (29%), insomnia (21%), cushingoid appearance (15%), and weight gain (15%). Other immunosuppressants were administered if glucocorticoids were contraindicated, if they were liable to cause unacceptable side effects, or in cases of an insufficient response. This occurred in 29.1% of patients (n = 39). Yet, of those patients, 13 received other forms of steroids (see also Table 3; budesonide [n = 6] at a median daily dose of 9.2 mg/day per participant, and methylprednisolone [n = 7] at a median daily dose of 29.4 mg/day per participant). The remaining 24 and 13 patients received tacrolimus (median daily dose of 4 mg/day per participant) and mycophenolate (median daily dose of 1,787 mg/day per patient), respectively. All grade 3 ALT elevations occurred within 36 weeks (9 within 26 weeks) following infusion and all resolved after glucocorticoid therapy. No event met Hy's law criteria for drug-induced liver injury (ALT >3x the upper limit of normal + total bilirubin >2x the upper limit of normal after excluding other causes)[58] and almost all events resolved following treatment (96.2%).

In the HOPE-B trial[40] the number of patients with ALT elevations was remarkably lower than in the GENEr8-1 trial: eleven patients (20%) developed post-baseline ALT elevations reported as AEs, with the majority being mild (three patients with grade 0, seven patients with grade 1). Only one patient experienced grade 3 ALT elevations. The time from infusion to first ALT elevation was earlier (median 5.1 weeks, with all ALT elevations occurring in the first 17 weeks) while duration of ALT elevation remained comparable (median 17 days in the HOPE-B trial). The majority of patients with an ALT elevation received glucocorticoids (n = 9/11) and the median duration of therapy was 74 days. No other immunosuppressive agents were given to control and resolve ALT elevations. No patient required steroid treatment beyond week 26 and no steroid-related AEs were reported. The recommended prednisone dose was 60 mg/day initially over 7 days, followed by a reduction of the drug to 40 mg/day in week 2, 30 mg/day in weeks 3 and 4 and then to a maintenance dose of 20 mg/day until transaminase levels returned to baseline levels. Then the daily dose of prednisone was reduced weekly by 5 mg/week. All ALT elevations resolved.

Table 2. Overview of the clinical presentation and management of ALT elevation in available phase III trials following liver-directed AAV gene therapy.

Study	GENEr8-1[37] n = 134	HOPE-B[40] n = 54
AAV vector	AAV5	AAV5
Patients with post-baseline ALT elevation reported as an AE, n (%)	115 (85.8)	11 (20)
Patients with highest grade ALT elevation, n (%)		
Grade 1	83 (61.9)	7 (13.0)*
Grade 2	21 (15.7)	0
Grade 3	11 (8.2)	1
Grade 4	0	0
Time from infusion to first ALT elevation, weeks median (min, max)	8.0 (1, 104)	5.1 (3.1, 17.1)
Duration of ALT elevation, days, Median (min, max)	15 (1, 488)	17.0 (5, 127)
Participants with per-protocol glucocorticoid use, n (%)	106 (79.1)	9 (16.7)
Duration of glucocorticoid therapy per participant, days, median (min, max)	230 (22, 551)	74 (51, 130)
Average daily glucocorticoid dose, per participant, mg/day, median (min, max)	33.3 (10, 80)	25.8 (21, 37)
Outcome of events, n (%)		
Resolved	306 (96.2)	11 (100)
Recovering	2 (0.6)	
Not recovered	9 (2.8)	
Unknown	1 (0.3)	

AAV, adeno-associated virus; ALT, alanine aminotransferase; SAE, serious adverse event.
*Additional three patients with grade 0 ALT elevations.

Table 3. Overview of other immunosuppressants used in the GENEr8-1 trial to control ALT elevations and immune-mediated host responses.

Study	ITT (n = 134)
Participants with any use of other immunosuppressant, n (%)	
Budesonide	6 (4.5)
Tacrolimus	24 (17.9)
Mycophenolate	13 (9.7)
Methylprednisolone (i.v./p.o.)	7 (5.2)
Median time from infusion to first other immunosuppressant use, weeks	21.3 (4, 70)
Median duration of use per participant, weeks	
Budesonide	127 (22, 329)
Tacrolimus	128 (34; 305)
Mycophenolate	241 (41, 367)
Methylprednisolone (i.v./p.o.)	106 (3, 290)
Median daily dose per participant, mg/d	
Budesonide	9.2 (5, 14)
Tacrolimus	4.0 (1, 14)
Mycophenolate	1,787 (1,105, 2,373)
Methylprednisolone (i.v./p.o)	29.4 (17, 1,000)

ALT, alanine aminotransferase; ITT, intention-to-treat; i.v., intravenous; p.o. per os.

Taken together, monitoring ALT closely in patients undergoing AAV gene therapy seems warranted: current guidelines recommend weekly monitoring within the first 6 months of gene therapy and monthly monitoring for 2 years thereafter.[59] Currently trials evaluating prophylactic immunosuppressive therapy in patients with haemophilia A with higher rates of drug-induced hepatitis are being conducted (*e.g.* GENEr8-3, NCT04323098). The feasibility and safety of longer ALT surveillance intervals in patients receiving gene therapies associated with low rates of drug-induced hepatitis or in less severe cases needs to be assessed in future studies.

Even closer follow-ups are necessary in cases with already detected moderate to severe ALT elevations and extended diagnostics should be performed (*e.g*. serum bilirubin, international normalised ratio, serum lactate dehydrogenase, alkaline phosphatase, gamma-glutamyltransferase, c-reactive protein, blood count etc.) to rule out other causes of ALT elevation including haemolysis, mechanic cholestasis or cholangitis/cholecystitis. Sonography or additional imaging may be required if clinically appropriate.

Prior to therapy, patients should be screened for concomitant liver disease (*e.g.* viral hepatitis, alcoholi-related or non-alcoholic steatohepatitis, autoimmune or cholestatic liver diseases). Viral hepatitis (note patients with haemophilia are at a higher risk of viral hepatitis due to prior blood transfusions or receipt of blood products) should be treated before the initiation of AAV gene therapy, as immunosuppressant therapy may be necessary and may worsen untreated hepatitis B or C. HBsAg-negative, anti-HBc-positive patients are at moderate risk (1-10%) of HBV reactivation if corticosteroids (>10 mg for >4 weeks) are administered.[60] Consecutively, current guidelines recommend monitoring these patients with HBsAg and/or HBV DNA every 1-3 months.[61] Of note, patients with advanced chronic liver disease or cirrhosis have been excluded from all phase III trials so far[32,37] and there is no data on the risk-benefit ratio in patients with advanced liver fibrosis, necessitating an individualised approach to therapy.

In a recent trial investigating the safety and efficacy of AAV8 gene therapy for Crigler-Najjar Syndrome, preemptive steroid therapy was applied, starting with 100 mg methylprednisolone (i.v.) followed by 40 mg prednisone orally, which was tapered beginning at week 3 and suspended at week 8.[62] While preliminary results are encouraging for this patient collective, preemptive steroid protocols may be an interesting option that warrants futher evaluation in the context of gene therapies associated with a high risk of related hepatitis or that target an underlying liver disease. The value of this approach in patients with haemophilia is the subject of an ongoing clinical trial and until this trial is completed and the data analysed it is not possible to provide a recommendation on the use of prophylactic steroids in patients receiving AAV-mediated gene therapy for haemophilia.

In the case of AAV gene therapy-associated ALT elevations where other causes have been ruled out, immunosuppressive therapy should be initiated, with the best data available for oral prednisone or prednisolone starting at 60 mg per day. Therapy should be tapered over a period of at least 8 weeks if transaminases normalise over the course of treatment. Alternate treatment options supported by available date (though in a limited number of patients) are budesonide, methylprednisolone (for severe cases), tacrolimus or mycophenolate. Of note, ALT elevations may occur more than once and patients should be monitored following successful treatment with immunosuppressive agents and retreated in the event of another event. The exact triggers that require an escalation of immunosuppression are not yet defined; to date, different approaches have been deployed and there is no consensus on when further immunosuppression should be deployed. The authors are of the opinion that most of the data in autoimmune hepatitis is derived from the use of azathioprine, usually starting at a dose of 0.5 to 2.0 mg/kg, and we therefore prefer the use of this agent and would consider introducing this drug if the ALT elevation has not begun to reduce significantly in the presence of a falling clotting factor level after at least 2 weeks of prednisolone therapy or in case of relapse during steroid tapering. An alternative option with the second best data available is mycophenolate motefil at a dose of up to 2,000 mg/day. It has been more commonly used as an alternative immunosuppressant in gene therapy studies, and in autoimmune hepatitis it is regarded as the second-line treatment of choice, with a recent promising trial (premliminary report) suggesting good efficacy as a first-line treatment as well.[37,63–65] In case of further worsing hepatitis, alternative diagnoses should, again, be excluded and an additional steroid dose escalation to up to 10 mg/kg methylprednisolone for a maxium of 3 days with a tapering protocol thereafter can be considered: This dose escalation regimen has been used for some prednisolone failures in gene therapy studies and is like that applied in cases of acute liver graft rejection.[37,66]

HCC risk in patients with AAV gene therapy

During early gene therapy, which often involved lentiviral vectors, genotoxicity – *e.g.* insertional mutagenesis and creation of oncogenic fusion proteins, remained a pivotal concern. Acute lymphocytic leukaemia, T-cell lymphoproliferative diseases, myelodysplasia and myeloid leukaemias were reported in early trials.[67–69] Since then, research has focused on altering promoter or enhancer sequences that were thought to be responsible for these genotoxic effects. Specifically, AAV is known to be largely nonintegrating into the host genome, as gene expression is achieved through the assembly of non-integrating episomes in the cell nucleus.[43] However studies in human liver cancers have shown that AAV2 clonal integrations can be detected in a small proportion of malignancies, raising the possibility that, in some circumstances, AAV2 may be implicated in oncogenesis.[70] When assessing the risk of HCC incidence after AAV gene therapy, important factors such as timing of delivery and vector dose should be accounted for. Additionally, selection of enhancers/promoters has to be considered, as some may activate adjacent genes, while others, even strong and liver-specific ones, probably do not.[21] In rodent models, liver tumours have been observed following transfection with AAV vectors, given in high doses and containing strong promoters, in neonatal or tumour-prone mouse strains.[21,71–77] Interestingly, the development of HCC was not

reported after AAV injection in animals older than 6-8 weeks.[78,79] In fact, a prospective trial in 6-8-week-old mice reported a low incidence of HCC, similar to the observed spontaneous HCC formation rate during a 2-year follow-up.[80] One mouse had a pathogenic integration of AAV when the tumor was analysed. In the same trial, no liver tumor formation was found in 50-day-old AAV-treated cats over a course of 8 years of follow-up after vector administration. Interestingly, all mice on a high-fat diet that were transfected with the AAV BDD-FVIII, which is prone to misfolding in the ER, developed HCC in a recent study.[81] In the same study, rates of HCC were much lower when mice were treated with a vector encoding for a BDD-FVIII variant that folds more efficiently. These findings raise concerns about the long-term effect of long-lasting transgene expression if protein misfolding occurs, as it may trigger chronic cellular stress in the context of a high-fat diet. These observations are particularly relevant due to the high prevalence of non-alcoholic fatty liver disease in developed and developing countries. Whether these observations are relevant to other aetiologies of chronic liver inflammation remains unknown. The authors concluded that limited transgene expression per hepatocyte and/or the use of proteins that avoid misfolding may enhance safety.[81]

Unfortunately, data on large clinical study populations are lacking. In 2020, a case report was published concerning a participant of the HOPE-B trial who had received etranacogene dezaparvovec to treat haemophilia B and developed a HCC during follow-up.[9] The patient was >65 years old and had a history of hepatitis C (eradicated 3 years prior to study inclusion) and hepatitis B, with evidence of non-alcoholic fatty liver disease on liver biopsy. Following surgical resection, tumour tissue was analysed and several commonly associated abnormalities on chromosomes 1 and 8, mutation of TP53 and several other potentially oncogenic genes were found. So far, findings from this investigation could not link AAV therapy to HCC development in this case.[82]

Taken together, patients with haemophilia have an increased risk of HCC development in comparison to the normal population.[83] Risk factors such as chronic liver disease caused by viral hepatitis are more common in these patients, while the role of AAV gene therapy remains uncertain in HCC development, with animal models presenting conflicting results. However, concerns have been raised in the context of concomitant chronic liver inflammation (*e.g.* high-fat diets). Until further data is available, we suggest patients should be screened for HCC prior to AAV therapy and be entered into surveillance programmes like those used in other chronic liver diseases, *i.e.* sonography and determination of alpha-fetoprotein every 6 months.

Conclusions

The licensing of gene therapies for people with haemophilia is an exciting therapeutic advance that has the potential to improve the lives of patients with clotting disorders. The major side effect of therapy is a transient hepatitis that may be associated with a reduction in treatment efficacy. The mechanisms underlying the ALT flares associated with gene therapy remain to be determined and the long-term risks associated with these new treatments is not yet known. It is however clear that optimal use of these new therapeutics will require close collaboration between haematologists, hepatologists and the community of patients in order to maximise the value and minimise the risks of these new treatments.

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Abbreviations

AAV, adeno-associated virus; AE, adverse event, ALT, alanine aminotransferase; EMA, European Medicines Agency; ER, endoplasmic reticulum; FDA, US Food and Drug Administration; FIX, factor IX, FVIII, factor VIII, ITR, inverted terminal repeats; SAE, serious adverse event; siRNA, small-interfering RNA.

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Conflict of interest

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Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

All authors contributed different sections of this article based on their expertise and MM collated and augmented prior to a final review by all authors.

Supplementary data

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