Gene therapy for hemophilia: what does the future hold?

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Abstract: Recent phase I/II adeno-associated viral vector-mediated gene therapy clinical trials have reported remarkable success in ameliorating disease phenotype in hemophilia A and B. These trials, which highlight the challenges overcome through decades of preclinical and first in human clinical studies, have generated considerable excitement for patients and caregivers alike. Optimization of vector and transgene expression has significantly improved the ability to achieve therapeutic factor levels in these subjects. Long-term follow-up studies will guide standardization of the approach with respect to the combination of serotype, promoter, dose, and manufacturing processes and inform safety for inclusion of young patients. Certain limitations preclude universal applicability of gene therapy, including transient liver transaminase elevations due to the immune responses to vector capsids or as yet undefined mechanisms, underlying liver disease from iatrogenic viral hepatitis, and neutralizing antibodies to clotting factors. Integrating vectors show promising preclinical results, but manufacturing and safety concerns still remain. The prospect of gene editing for correction of the underlying mutation is on the horizon with considerable potential. Herein, we review the advances and limitations that have resulted in these recent successful clinical trials and outline avenues that will allow for broader applicability of gene therapy.

Keywords: adeno-associated virus, gene therapy, hemophilia, lentivirus

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Introduction

Hemophilia is an X-linked bleeding disorder caused by deficiency of coagulation factor VIII (FVIII) or factor IX (FIX) function due to mutations in the *F8* or *F9* gene, respectively.[1] FVIII deficiency, or hemophilia A (HA), accounts for 80% of cases and affects 1:5000 male births while FIX deficiency, or hemophilia B (HB), affects 1:30,000 male births worldwide.[1] In both disorders, bleeding severity correlates with residual factor activity wherein patients who have less than 1% activity (severe disease) present with frequent, spontaneous hemorrhages in joints (hemarthrosis), soft tissue, and muscles. Bleeding may also occur into closed spaces (e.g. intracranial or retroperitoneal), which can be life threatening. Patients with moderate disease (1–5% activity) only rarely have spontaneous bleeds, and those with mild disease (5–30% activity) generally only present with trauma-induced or postsurgical bleeding.[1] The current standard of care for patients with severe disease is initiation, in childhood, of lifelong prophylaxis with exogenous factor replacement to limit spontaneous bleeds in an effort to curtail morbidity and mortality.[2] However, in the United States, only around 60% of young adults and adults report adherence to prophylaxis due to its inherent complexity,[3] resulting in unacceptable rates of bleeding and long-term joint complications. Further, challenges of intravenous factor administration in young patients and lack of access to factor concentrates in developing countries pose additional barriers to optimal prophylaxis administration.

Cloning of the *F8*[4] and *F9*[5] genes not only allowed for the generation of recombinant clotting factors[6,7] for therapy but also instigated gene therapy efforts to, at first, ameliorate phenotype or give simplified prophylaxis and, more recently, potentially cure

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disease. Hemophilia is an optimal target for gene therapy due to the monogenic nature of inheritance and the observation that even minimal increases in clotting factor activity can significantly improve quality of life.[1] Lessons from early gene therapy trials[8,9] have been integrated into recent successful early phase clinical trials for both HA and HB.[10–14] Although gene therapy targeting the liver has come a long way, certain limitations preclude its ability to become a definitive cure for all patients; overcoming these will be of utmost importance in the coming years.[15] Here, we review the advances that have resulted in recent successful phase I/II clinical trials and outline avenues that will allow for broader applicability of gene therapy. In particular, we focus on the needs of patients with pre-existing neutralizing alloantibodies to the vector or the clotting factors (clinically termed inhibitors), those with liver disease, and pediatric patients.

Recent successes in gene therapy for hemophilia

Decades of preclinical and clinical work have resulted in the current successful phase I/II gene therapy trials for hemophilia. All published and currently open studies use adeno-associated viral (AAV) vectors.[8–14] AAV is a replication defective member of the parvovirus family and is nonpathogenic in humans.[15] The vector genome is manipulated to replace the AAV coding sequences with the transgene of interest under the control of a tissue-specific promoter, such as in liver gene therapy, to yield a recombinant AAV (rAAV) vector (Figure 1). rAAV is a nonintegrating vector and the vector genome is maintained episomally in transduced cells. The size of the AAV genome (~4.7 kb) limits the size of the packaged transgene,[15] such that the *F9* cDNA at 1.6 kb[5] was easier to incorporate than the 7 kb *F8* cDNA.[16] Even after removal of the *F8* B domain (~2.6 kb), which is not required for coagulation function,[16] incorporation of *F8* cDNA has been accomplished slowly by a combination of distinct strategies.[17,18] Thus, early studies focused on HB given the smaller size of the *F9* gene, despite its lower prevalence.

In an early AAV clinical study with percutaneous injection of AAV2-FIX into skeletal muscle, the levels of neutralizing antibodies (NAbs) to AAV2 did not preclude local gene transfer or FIX transgene expression.[9] Thus, in the subsequent first liver-directed gene therapy trial for HB, the presence of NAbs to AAV was not listed in the exclusion criteria.[8] This trial demonstrated the ability to achieve therapeutic FIX levels in the highest dose cohort [2×10^{12} vector genomes/kg (vg/kg)] using AAV2 and a wild-type FIX transgene (FIX-WT).[8] The low and intermediate dose cohorts (8×10^{10}–4×10^{11} vg/kg) were designed as subtherapeutic doses to assess safety. The first patient in the high-dose cohort achieved a FIX level of around 12% but then developed a cellular immune response against the AAV capsid, as noted by a transient increase in liver enzymes and decrease in transgene expression.[8] In contrast to the intramuscular trial, the second patient's transduction efficiency was hindered by pre-existing NAbs to the AAV capsid.[8] Pre-existing NAbs can be present in 30–70% of the general population for a given AAV serotype.[19] Subsequent liver-directed trials, therefore, excluded patients with pre-existing NAbs to the trial AAV serotype and planned for immunosuppression with steroids if liver enzymes increased or factor expression decreased in order to limit the capsid-triggered cellular immune response.[10–14,20] All studies to date have used an AAV serotype with liver tropism utilizing a codon optimized transgene under the control of a liver-specific promoter.[8,10–14,20] Differences in AAV serotype, CpG content of the transgene, vector inverted tandem repeats (ITRs), presence of empty capsids as byproducts, and manufacturing cell line comprise the major differences between products[21] (Figure 1, Tables 1 and 2). Some trials are utilizing hyperactive transgenes in an effort to decrease vector dose, which has been successful.[10]

AAV liver-directed HB clinical trials

Table 1 provides a summary of recently completed and open AAV-based trials for HB. The initial AAV2-FIX-WT trial by Manno and colleagues infused patients *via* the hepatic artery to enhance hepatocyte transduction.[8] AAV8, compared with AAV2, has higher liver-specific tropism allowing for peripheral infusion of the vector.[13,14] Building on the lessons from the AAV2 trial, the next HB study sponsored by St Jude Children's Research Hospital (SJCRH) and University College of London (UCL) infused AAV8-FIX-WT in 10 subjects at doses between 2×10^{11} and 2×10^{12} vg/kg and excluded patients with anti-AAV8 NAbs.[13,14] This study demonstrated sustained FIX activity of 3–5%, in a dose-dependent manner, with no long-term safety issues identified at 3 years, with ongoing follow up.[13,14] However, four of six subjects in the

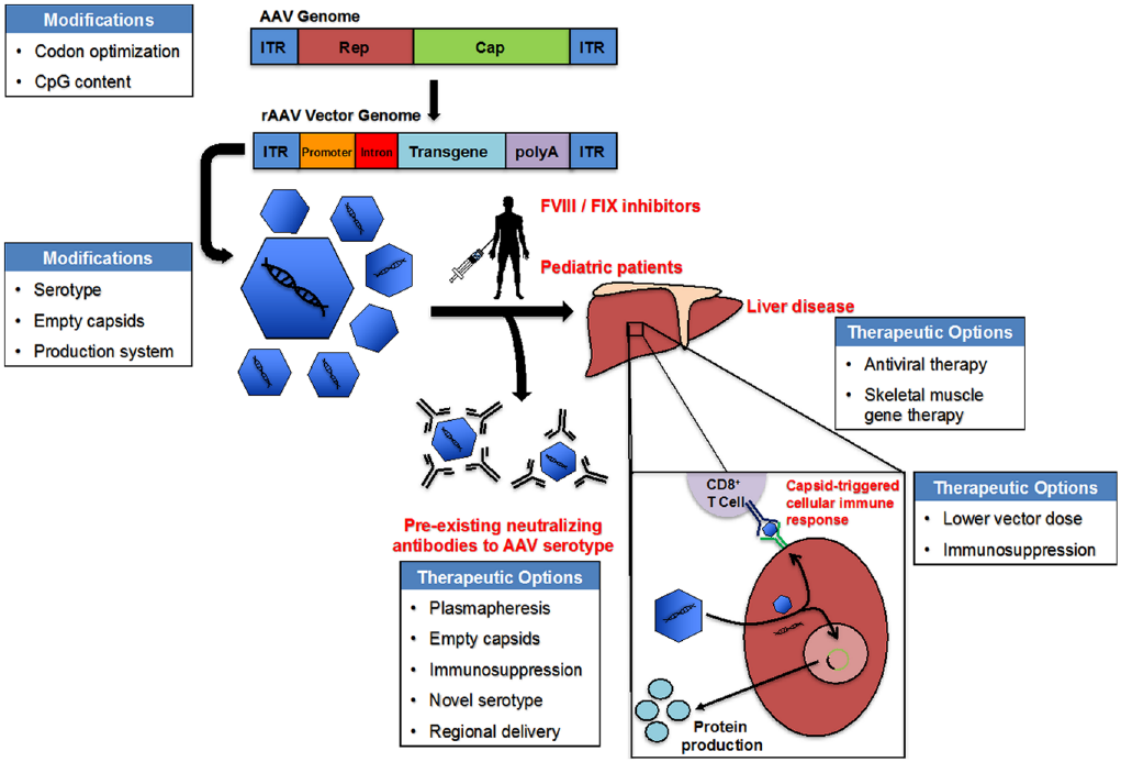

Figure 1. Overview of adeno-associated virus (AAV) mediated liver-directed gene therapy for hemophilia. The wildtype AAV genome consists of two inverted tandem repeat (ITR) regions flanking the *rep* (replication) and *cap* (capsid) genes. These genes are replaced by a tissue-specific promoter with enhancer, intron, and transgene of interest in the recombinant (r)AAV vector genome, which is packaged into capsids and injected into subjects *via* a peripheral venous infusion. Once infused, rAAV vector can be neutralized by pre-existing antibodies in a serotype-specific manner or transduce hepatocytes where the capsid is degraded and the genetic material maintained as an episome in the nucleus to produce the transgene product. Capsid peptides can be presented on the surface of hepatocytes to CD8+ T cells, thought to lead to a cellular immune response coinciding with loss of transgene and rise in liver transaminases in some clinical trials. Modifications in the transgene, serotype, infusion of empty capsids, and production process may all affect efficacy. Options to bypass the pre-existing humoral response or liver disease are listed. Additional hurdles to general application of liver-directed AAV gene therapy include inhibitors to factors VIII and IX as well as infusion in young patients. FVIII, factor VIII; FIX, factor IX; CpG, cytosine-guanine residues.

high-dose cohort had elevated liver transaminases concomitant with a positive T-cell enzyme-linked immunospot (ELISPOT) assay against the AAV capsid, signifying a capsid-triggered cellular immune response [Figure 2(a)]. Gaining from the experience of the earlier AAV2 study,[8] this study was the first to identify that prompt initiation (<48 h) of steroids in the presence of increased liver enzymes or drop in transgene expression levels prevented further loss of FIX activity.[13,14] In the follow-up study with an additional four patients infused in the high-dose cohort, the annualized bleed rate (ABR) dropped from a median of 16.5 to 1 (94%) and overall ABR decreased from 15.5 to 1.5 (90%) in a vector dose-dependent manner.[13,14]

The observation that there is vector dose dependency on the rate and presence of immune response to the vector capsid prompted the development of strategies to allow lower therapeutic vector doses. The identification of a naturally occurring hyperactive FIX variant (FIX-Padua), which yields approximately eightfold increased specific activity,[25] afforded the opportunity to achieve therapeutic FIX levels with a lower vector dose. Our laboratory reported success with this strategy in inhibitor-prone HB dogs treated with AAV8 carrying canine (c) FIX-Padua at $1–3 \times 10^{12}$ vg/kg with therapeutic FIX levels of 25–200%.[26] There was no clinical or laboratory evidence of thrombosis and no antibody to the transgene after recombinant cFIX-WT infusion. In these dogs,

Table 1. AAV gene therapy trials for hemophilia B.

Sponsor	Serotype	Transgene	Highest dose (vg/kg)	Mean FIX:C (%)	T-cell ELISPOT	↑ ALT	↓ FIX:C with ↑ ALT	Steroid treatment	Status[$]	Ref.
CHOP/UPENN	rAAV2	FIX-WT	2×10^{12}	12*	N/A	1/2	1/2	0/2	Closed	8
UCL/SJCRH	rAAV8	FIX-WT	2×10^{12}	5.1	4/6	4/6	4/6	4/6	Recruiting	13,14
Shire	rAAV8	FIX-Padua	3×10^{12}	0				2/3	Closed	20,22
Spark Therapeutics	Spark100	FIX-Padua	5×10^{11}	33	2/10	2/10	2/10	2/10	Closed	10
uniQure	rAAV5	FIX-WT	2×10^{13}	6.9	0/5	2/5	0/5	2/5	Closed	11
Dimension	rAAV-rh10	FIX-WT	5×10^{12}	6.7	2/3	3/3	3/3	3/3	Closed	23
Sangamo Therapeutics	rAAV6	Zinc finger-FIX	N/A	N/A	N/A	N/A	N/A	N/A	Recruiting	N/A
Freeline Therapeutics	rAAV-engineered	FIX-Padua	N/A	N/A	N/A	N/A	N/A	N/A	Not yet recruiting	N/A

*One subject had peak activity of 12% before immune response and subsequent decline to less than 1%.
[$]Status determined by data available to ClinicalTrials.gov as of March 2018.
AAV, adeno-associated virus; ALT, alanine aminotransferase; CHOP, Children's Hospital of Philadelphia; ELISPOT, enzyme-linked immunospot; FIX, factor IX; N/A, not available; rAAV, recombinant adeno-associated virus; Ref., reference; SJCRH, St Jude Children's Research Hospital; UCL, University College of London; UPENN, University of Pennsylvania; WT, wild type.

Table 2. AAV gene therapy trials for hemophilia A.

Sponsor	Serotype	Transgene	Highest dose (vg/kg)	Mean FVIII:C (%)	T-cell ELISPOT	↑ ALT	↓ FVII:C with ↑ ALT	Steroid treatment	Status*	Ref.
Biomarin Therapeutics	rAAV5	BDD-FVIII	6×10^{13}	4–270	0/7	7/7	1/7	7/7	Recruiting	[12]
Spark Therapeutics	Spark200/ LK03	BDD-FVIII	2×10^{12}	N/A	N/A	N/A	N/A	N/A	Recruiting	[24]
Shire	rAAV8	BDD-FVIII	N/A	N/A	N/A	N/A	N/A	N/A	Recruiting	N/A
Sangamo Therapeutics	rAAV2/6	BDD-FVIII	N/A	N/A	N/A	N/A	N/A	N/A	Recruiting	N/A
UCL/SJCRH	rAAV8	BDD-FVIII	N/A	N/A	N/A	N/A	N/A	N/A	Recruiting	N/A

*Status determined by data available to ClinicalTrials.gov as of March 2018.
AAV, adeno-associated virus; ALT, alanine aminotransferase; BDD-FVIII, B-domain-deleted factor VIII; ELISPOT, enzyme-linked immunospot; FVII, factor VII; FVIII, factor VIII; N/A, not available; rAAV, recombinant adeno-associated virus; Ref., reference; SJCRH, St Jude Children's Research Hospital; UCL, University College of London.

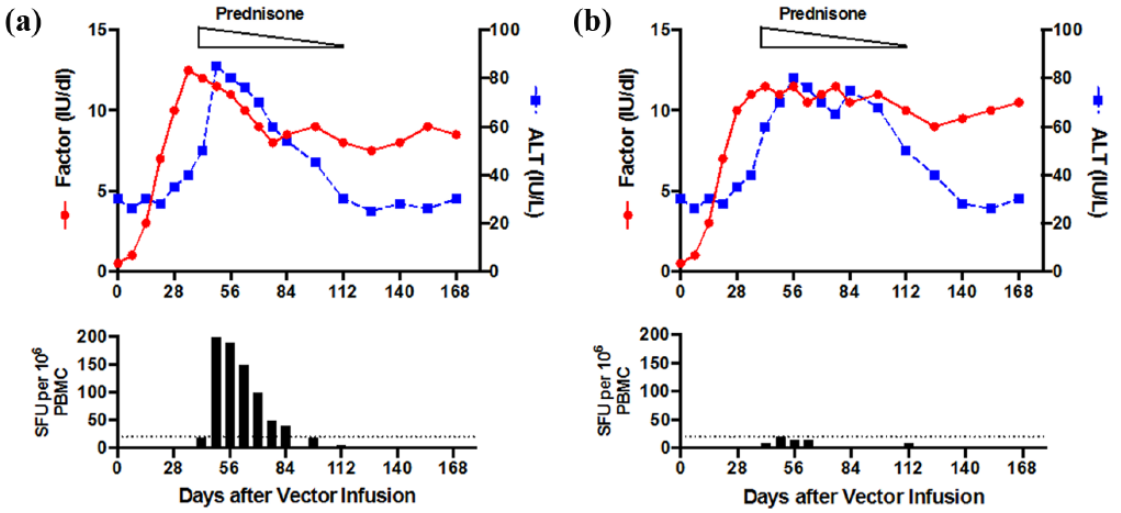

Figure 2. Modeling of capsid-triggered cellular immune response, liver transaminase, and factor level correlation in published adeno-associated virus (AAV) liver-directed hemophilia gene therapy clinical trials. Correlation between alanine aminotransferase (ALT, ■), factor activity (●) and T-cell enzyme-linked immunospot (ELISPOT). (a) The observation of a rise in liver alanine aminotransferase (ALT) with coincident decline in transgene expression is thought to be associated with a cellular immune response against the AAV capsid as confirmed by positive ELISPOT (bottom panel). This effect can be rescued with a short course of corticosteroids (prednisone). (b) In two recent trials, a rise in ALT does not always correlate with loss of factor activity or positive T-cell ELISPOT. Although steroids are used in these trials, ALT response is not uniform in all cases.
PBMC, peripheral blood mononuclear cells; SFU, spot-forming units

cFIX-Padua specific activity was similar to that of FIX-Padua in the original family.[26]

Subsequently, a clinical study by Shire (Dublin, Ireland) utilizing AAV8-FIX-Padua at doses of 2×10^{11} to 3×10^{12} vg/kg demonstrated long-term expression of FIX at 20% in a single subject infused with 1×10^{12} vg/kg without development of inhibitors to the transgene.[22] However, in the mid- and high-dose cohorts, after near normal FIX activity, immunosuppression with prednisone did not prevent loss of transgene expression caused by the cellular immune response to the capsid.[22,27] In the study sponsored by Spark Therapeutics, (Philadelphia, PA, USA) a novel AAV serotype (Spark100) with FIX-Padua was infused in 10 subjects at a fixed dose of 5×10^{11} vg/kg and resulted in sustained FIX activity of around 30% with lower rates of immune response to the vector capsid.[10] Only two of 10 subjects required steroids compared with four of six in the SJCRH/UCL trial. Notably, these patients retained FIX levels within the mild deficiency to normal range even after prednisone was discontinued (30–80%). All patients have stopped prophylaxis with a concurrent decrease in ABR from 11.1 to 0.4 (96%) and there has been no evidence of inhibitor formation to FIX-Padua.[10] The planned phase III trial utilizing this vector will be sponsored by Pfizer (New York, NY, USA).[28]

The trials mentioned above used AAV vectors generated in mammalian cells using triple transfection of plasmid DNA for vector production.[8,10,13,14,20] The uniQure-sponsored HB trial utilized AAV5-FIX-WT generated in an insect cell line using a baculovirus production system at doses of 5×10^{12} and 2×10^{13} vg/kg, with five subjects in each cohort.[11] Results show mean steady-state FIX activity of 3–12% with a decrease in spontaneous ABR from 9.8 to 4.6 (53%) in the low-dose cohort and from 3 to 0.9 (70%) in the high-dose cohort.[11] However, there was not a clear linear dose response to the vector, as despite a fourfold higher vector dose there was only 1.6-fold higher FIX activity.[11] Furthermore, although three of ten subjects had an increase in alanine aminotransferase (ALT) and received steroids, this was neither associated with anti-capsid cellular immune response nor did it have an effect on FIX activity[11] [Table 1, Figure 2(b)], in contrast to the other studies.[8,13,14,22] The underlying mechanism of this complication is unknown. Of note, the phase III study announced by uniQure (Amsterdam, Netherlands) will use AAV5-FIX-Padua instead of wild type (WT)

transgene.[11] The trials to date have clearly demonstrated that FIX-Padua is likely the optimal transgene to ameliorate the disease phenotype as well as decrease the likelihood of a capsid-triggered immune response by allowing lower vector doses. FIX-Padua has no increased immunogenicity in preclinical models, and in these models, gene therapy with FIX-Padua can actually eradicate pre-existing inhibitors to FIX-WT.[26]

AAV liver-directed HA clinical trials

Ongoing and published AAV-based clinical trials for HA are summarized in Table 2. HA gene therapy was initially limited by the large size of the *F8* gene; however evidence that removal of the B domain, which comprises 40% of the gene, does not affect coagulation function[16] as well as further vector optimization have resulted in successful generation of B-domain-deleted (BDD) FVIII AAV vectors for clinical trials.[17,18] Biomarin (Novato, CA, USA) has reported success with its HA gene therapy trial utilizing rAAV5 with BDD-FVIII at doses of 2×10^{12} to 6×10^{13} vg/kg[12]. Surprisingly, in the low (2×10^{12} vg/kg) and intermediate (2×10^{13} vg/kg) dose cohorts, FVIII expression was either unchanged or only 1–3% (n = 1/dose).[12] In the high-dose cohort (n = 7), the first treated subject had an increase in ALT (1.5 fold baseline but below the upper limit of normal) between weeks 4 and 7, prompting the use of steroids for a presumed capsid-triggered immune response. All subsequent patients in the high-dose cohort were treated prophylactically with steroids. As in the AAV5-FIX uniQure trial,[11] in the Biomarin trial there was no clear evidence of a connection between increased ALT, anti-capsid T-cell response, steroid use, and FVIII activity [Table 2, Figure 2(b)]. In four of the seven patients on high-dose treatment, steroid therapy did not halt ALT elevation. Normalization of ALT with respect to steroid discontinuation varied widely, with some patients having increases even after steroids were stopped. The steroid dose was titrated down as per the protocol in all patients. Prior studies have shown correlation between ALT and capsid-triggered immune response[8,10,13,14] but no capsid-directed T-cell response was seen in these HA subjects, raising the question of whether the ALT elevation seen here is not an immune response but rather potential hepatocyte toxicity or other unknown effects.[12] The exact mechanism behind this outcome is not completely understood and remains under investigation but raises the question of whether the insect-line, baculovirus system manufacturing process, or the AAV5 immune response may differ from other rAAV vectors produced in mammalian lines using plasmid DNA. Over a 12-month observation period, ABR decreased 94% from 16 to 1 and the reported FVIII activity ranged between 19% and 164% without a linear dose response (threefold higher dose between cohorts with 10- to 80-fold increased FVIII activity).[12] The safety of chronic supraphysiologic FVIII activity is unknown and may carry a risk of thrombosis;[29,30] furthermore, these levels are unnecessary for phenotypic improvement. Long-term follow-up data from these subjects may guide the efficacy and further understanding of the mechanism of this strategy. A phase III trial is planned using vector doses from 4×10^{13} to 6×10^{13} vg/kg.[12]

Spark Therapeutics has also reported early results from its phase I/II clinical trial using a novel AAV serotype (Spark200/LK03[31]) with BDD-FVIII at doses of 5×10^{11} (low dose), 1×10^{12} (intermediate dose), and 2×10^{12} vg/kg (high dose). The two patients each treated at the low and intermediate dose had steady-state FVIII levels of 10–13% and 9–13%, respectively.[24] The first intermediate dose subject (1×10^{12} vg/kg) had increased ALT and was started on immunosuppression with stable FVIII activity thereafter, and the second subject was treated with steroids empirically. Data from the high-dose (2×10^{12} vg/kg) subjects have not yet been released. As in the HB trials, there is wide variation in vector dose between these HA trials (~60-fold higher vector dose for Biomarin compared with Spark HA trial), and whether one strategy may be more successful remains to be determined. There are also concerns about manufacturing feasibility, and the short duration of follow up of these recent trials precludes definite safety assessment at this point.

Future and challenges of gene therapy

The variety of AAV serotypes, the wide range of therapeutic vector doses, and differing manufacturing processes make a comparison of these products very difficult. However, the common threads amongst these trials are the following exclusion criteria: patients under 18 years of age; patients with less than 30–50 exposure days to FVIII/FIX concentrates; underlying liver disease or active viral hepatitis B or C infections (HBV/HCV); presence of NAbs to AAV serotype higher than a cutoff; and current or prior history of inhibitors to FVIII or FIX. Despite promising results, due to the limits imposed by these exclusion criteria, gene therapy is not yet at the point of

being universally applicable to all persons with hemophilia. Notably, in the United States, like many other countries, the high prevalence of HCV in patients with hemophilia over the age of 35 (in 2018) means that over 35% of adult patients who could benefit from gene therapy will be ineligible for these liver-directed approaches.[3] What impact, if any, the ability to cure HCV with new antivirals will have remains to be seen.

The ability to mitigate pre-existing NAbs to AAV will also expand the eligible population as around 30–70% of patients have NAbs to specific serotypes.[19,32,33] However, attempts to eliminate pre- existing NAbs utilizing either immunosuppression,[34,35] plasmapheresis,[36] inclusion of empty capsids[37] to serve as decoys, novel bioengineered capsids, or localized vector infusion[38] have only modestly decreased titers and not eliminated the NAbs. As expected, the development of a robust serotype-specific humoral response after vector infusion would preclude reinfusion with the same vector should response wane either due to capsid-triggered T-cell response or dilution of transduced hepatocytes with liver growth and increased blood volume, as when the vector is delivered to young patients.[15]

Finally, up to 30% of patients with severe HA and 5% of patients with severe HB develop inhibitors to the infused protein,[39] which decrease or nullify achievement of hemostasis with replacement protein. Currently, the only known curative strategy for inhibitors is infusion of frequent, high doses of factor concentrates over a period of months to years termed immune tolerance induction (ITI), which is moderately (~60%) successful in patients with favorable risk characteristics.[40] The goal of ITI is to allow the use of FVIII/FIX protein for replacement therapy as they more effectively achieve hemostasis than alternative bypassing agents (BPAs), such as activated prothrombin complex concentrates (aPCCs) or recombinant factor VIIa (rFVIIa),[41] which circumvent the need for FVIII and thereby provide alternate pathways for generation of thrombin. While ITI is more cost effective than life-long BPA therapy for hemostasis, it comes at great economic burden (~$1 million for a 20 kg patient) and is demanding, as evidenced by around 20% of patients withdrawing from participation in the International ITI (I-ITI) study.[40,42,43] There is mounting preclinical evidence in HA/HB dogs and mice[26,44–47] that AAV liver-directed gene therapy in animal inhibitor models is tolerance inducing and successful application to this population would result in dramatic improvement in quality of life for these patients. Efforts to overcome these obstacles should broaden the availability of gene therapy for the vast majority of patients with hemophilia.

Gene therapy for eradication of inhibitors and immune tolerance induction

Inhibitors to FVIII and FIX are the most significant and challenging clinical complication to replacement therapy at the moment,[48,49] Inhibitors are measured in Bethesda units (BU), where 1 BU is defined as the amount that neutralizes 50% of the normal clotting factor activity. Patients with high-titer inhibitors (BU >5) are generally treated with BPA instead of FVIII/FIX concentrates to induce hemostasis. Despite the initiation of ITI in the 1970s, a prospective study (I-ITI), was only recently completed in 2012.[40] This study identified the characteristics of patients who were more likely to achieve tolerance as follows: BU less than 10 at start of ITI; historical peak BU less than 200; young age; and short time between diagnosis and ITI start.[40] While around 60% of patients with these favorable risk factors will achieve tolerance with ITI, there are not good options for those with refractory or relapsed inhibitors.[50] Furthermore, the time to reach BU less than 10 ranged from 3 to 8 months in I-ITI, and a subset of patients were unable to be randomized in the trial due to persistent BU greater than 10.[40] Prior attempts at immunosuppressive regimens alone or coupled with ITI have had mixed and modest success at inhibitor eradication.[51,52] Alternative strategies that simplify ITI are clinically necessary and could provide tremendous benefits to inhibitor patients. Indeed, the preclinical data below support the hypothesis that liver-directed gene therapy can provide the dual benefits of both eradicating inhibitors to the clotting factor *and* providing simplified prophylaxis thereafter.[53]

While several animal models of hemophilia have been characterized,[54] the canine model[55] is unique for several reasons: dogs naturally have hemophilia resulting from a spectrum of genetic mutations similar to humans; the disease phenotype is similar to humans; compared with mice, the colonies are outbred and of various genetic (and thereby immune) backgrounds; and characterization of cFVIII[56] and cFIX[57] cDNA affords the ability to produce recombinant cFVIII/cFIX protein for species-specific

Table 3. Canine models of hemophilia.

	Mutation	Inhibitor risk*	Nomenclature
HA			
Queens University	Intron-22 inversion	High	QU HA
University of North Carolina	Intron-22 inversion	Low/high[$]	UNC-CH HA
HB			
University of North Carolina	Missense	Low	UNC-CH HB
University of Alabama	Premature stop	High	UAB HB

*Risk of inhibitor development to canine FVIII/FIX.
[$]Initial colony did not develop inhibitors until introduction of an outside male breeder.
HA, hemophilia A; HB, hemophilia B; FVIII, factor VIII; FIX, factor IX.

immunologic studies.[53] The canine HA and HB models differ in their backgrounds, genetic mutations, and predisposition to antifactor immune responses (Table 3). While the HA dogs from Queens University (QU)[58] and University of North Carolina (UNC-CH)[59] both have mutations similar to the human *F8* intron 22 inversion, the QU HA dogs were initially more inhibitor prone after exposure to cFVIII. Subsequent introduction of an outside male breeder to the UNC-CH HA colony has created a subset of inhibitor-prone HA dogs there as well.[46] Murine hemophilia models, in contrast, are generally limited to two strains with very similar mutations, and immune studies are complicated by infusion of human xenoprotein.[60] Nonetheless, in both canine and murine models, mounting preclinical data demonstrate that consistent endogenous expression of FVIII/FIX after liver-directed gene therapy can result in transgene-specific immune tolerance and eradicate pre-existing inhibitors.[26,46,61–66] In contrast, collective data from nonhuman primates (NHPs) in AAV liver gene therapy for HB or HA resulted in inhibitor formation due to the use of a human transgene and precluded the consideration of transgene-specific ITI by gene therapy.[17] In the canine and murine liver-directed gene therapy studies, it was demonstrated that tolerance occurs through induction of a regulatory T-cell (Treg) response and other mechanisms.[46,61,62] Further evidence supporting the role of Tregs comes from a NHP study where the use of an anti-CD25 antibody, which depletes both T effector and Tregs, during the early phase of AAV2 liver-directed FIX expression resulted in inhibitor formation.[67]

The exact mechanism behind ITI by frequent exposure of FVIII/FIX protein concentrates is not completely understood, but the rationale is that prolonged and consistent antigen exposure would tilt the balance towards tolerance. The advantage of gene therapy for ITI is the opportunity to provide this continuous antigen endogenously with a single infusion.[68] Our laboratory investigated whether gene therapy could eradicate pre-existing inhibitors and thereafter allow for simplified prophylaxis with endogenous FVIII/FIX production. Within the UNC-CH HA inhibitor prone model, three dogs with historically high titers were able to eradicate their inhibitors 4–5 weeks after liver-directed AAV gene therapy with progressively increasing FVIII levels and improvement in the bleeding phenotype. In addition, they demonstrated normal pharmacokinetics to infused cFVIII recombinant protein thereafter,[46] in line with criteria for successful tolerance from I-ITI. A QU-HA dog who had developed inhibitors after prior exposure to human FVIII, which can cross react with cFVIII, given the same therapy had an anamnestic response that peaked at 216 BU but achieved tolerance by 18 months post infusion.[46] In fact, in ITI an anamnestic response greater than 200 BU was negatively correlated with tolerance induction.[69] Although this dog took longer to achieve FVIII tolerance, consistent endogenous antigen exposure *via* gene therapy was ultimately successful at eradicating the pre-existing inhibitor to both human and canine FVIII (unpublished observation, V.R.A. and Finn and colleagues[46]). In the canine HA inhibitor model, we have also noted an increase in Tregs with liver-directed gene therapy that precedes antibody eradication,[46,68] whereas in dogs

without inhibitors the Treg population does not change after gene therapy (unpublished observation, V.R.A.). Subsequent studies showed that administration of liver-directed AAV-cFVIII transgene in eight naïve UNC-CH HA dogs with intron 22 inversion resulted in only transient low-titer inhibitor formation in one dog, who was subsequently discovered to be a member of the inhibitor-prone subcolony.[70] This dog achieved FVIII tolerance without further intervention and all eight remained without inhibitors even upon subsequent protein challenges.[70]

Inhibitors to FIX are less common, occurring in only 3–5% of patients with severe HB, but have a higher prevalence in patients with large gene deletions or early stop codon compared with other mutations.[39] FIX inhibitors are more problematic clinically as they can be associated with allergic reactions to protein and nephrotic syndrome, posing additional challenges for ITI.[71,72] Moreover, ITI is effective in only around 30% of the cases in patients with HB. Our group has investigated whether liver-directed gene therapy may promote transgene tolerance in HB as well. With liver-directed gene therapy, HB dogs showed long-term expression of cFIX-WT.[63,64] We further demonstrated that AAV liver gene therapy with cFIX-Padua resulted in FIX activity of 25–40% and did not result in anti-FIX antibody formation in two inhibitor-prone University of Alabama (UAB) HB dogs.[26] Furthermore, a third dog with pre-existing inhibitor to cFIX-WT had a transient anamnestic response followed by subsequent spontaneous eradication of the inhibitor with FIX activity around 200% after ITI with cFIX-Padua gene therapy.[26] An additional dog with inhibitor to cFIX-WT was also tolerized with cFIX-Padua gene therapy (unpublished observation, V.R.A.). Thus, liver-directed gene therapy with FIX-Padua may be able to induce tolerance for patients with pre-existing anti-FIX-WT inhibitors as well.

Combined, these studies suggest that liver-directed gene therapy with AAV vectors can tilt the immune system towards transgene-specific tolerance. Inhibitors are most likely to develop in young patients with severe hemophilia, typically within their first 50 exposure days.[73] ITI *via* protein infusion or BPA prophylaxis in these patients may require the placement of a central venous catheter, which increases the risk of infectious and thrombotic complications.[74] Young patients may also need central venous catheters for routine FVIII/FIX prophylaxis. Furthermore, ITI takes several months to years to induce tolerance,[15,40] and patients can have several breakthrough bleeds leading to significant joint damage. Although recent developments such as emicizumab,[75] a bispecific antibody that mimics FVIIIa function by binding FIXa and FX, and inhibitors of natural anticoagulants[76–78] such as fitusiran, a small interfering RNA to antithrombin, may improve hemostasis in patients with inhibitors,[21] this still ties them to life-long therapy and does not eradicate the inhibitor in order to allow the use of factor for breakthrough bleeds,[75,76] which is the main goal of ITI. Given the inability to predict exact prohemostatic effects from these drugs, they may pose thrombotic risks[21] when combined with other clotting factors for bleeding, as evidenced by thrombosis (including thrombotic microangiopathy) in five patients in the emicizumab trial[75] when treated concurrently with aPCC for more than 24 h due to bleeding and one patient in the fitusiran trial[79] whose cavernous sinus thrombosis was initially thought to be a subarachnoid hemorrhage and received several doses of FVIII. Thus, likely the best therapeutic choice for a clotting factor deficiency is replacement of the clotting factor[41] as the ideal replacement or BPA therapy, and dosage in the setting of these newer agents remain unclear. The tremendous physical, financial, and psychosocial morbidity[80–83] for inhibitor patients can be significantly ameliorated if gene therapy is able to eradicate their inhibitors with a one-time infusion. Moreover, the presence of inhibitor in adolescent boys is also associated with delayed growth and maturation,[84] thus it is imperative to eradicate inhibitors rather than just using BPAs. As clinical gene therapy trials move into phase III studies, a powerful and meaningful next step would be to include patients with inhibitors as this can provide the *dual* benefits of eradicating the inhibitor and improving the disease phenotype with one infusion.

Patients with underlying liver disease

Prior to the advent of recombinant factors, patients with hemophilia were infused with pooled cryoprecipitate or plasma-derived factors.[1] The first evidence of liver injury in people with hemophilia treated with these products emerged in the 1970s with asymptomatic elevated transaminases documented in 45% of patients,[85] which persisted for several years and was found in the 1980s to cause progressive cirrhosis. Although

HBV was known to play a role, the identification of HCV as a contributor to this phenomenon did not occur until 1987.[86] Recent epidemiological studies from hemophilia treatment centers in the United States demonstrate that although no cases of HCV have been documented in patients born after 1992, approximately 35% of patients born in the US between 1983 and 1992 have HCV and the prevalence is greater than 80% for patients born between 1958 and 1982.[3] For safety concerns, clinical gene therapy trials have excluded patients with concomitant HBV or HCV infection, or liver disease from participation in early phase trials. Whether and how to include patients who have successfully been treated with anti-HCV antivirals remains an unsolved issue. New direct-acting antivirals demonstrate over 90% sustained virologic response, even in patients with advanced HCV liver disease, but whether the risk of hepatocellular carcinoma in these patients decreases will require further study.[87,88] Safety of liver-directed gene therapy in these patients is unknown.

Another safety issue is the concern for both acute and long-term hepatic genotoxicity from AAV vectors themselves. Studies have suggested a protective role of WT AAV in cancer.[89–91] Indeed, athymic nude mice injected with a colon cancer cell line treated with WT AAV2 had decreased tumor growth due to selective apoptosis induced by AAV in cells that lack activity of the tumor suppressor p53.[89] Preclinical studies have assessed the risk of genotoxicity from hepatocyte transduction with AAV vectors.[92] Despite the fact that AAV vector is nonreplicative and nonintegrating, studies in mice have shown that some integration can occur[93] and has been demonstrated to cause hepatocellular carcinoma (HCC) in neonatal mice given rAAV gene therapy[94,95] due to integration at a transcriptionally active *Rian* locus in neonatal mice, which is not present in humans.[96] Studies in older rodents, dogs, and NHPs[63,97–101] did not demonstrate increased HCC with AAV gene therapy. A comprehensive evaluation found that the HCC risk can be mitigated by modulating both vector characteristics (dose, promoter, enhancer) and subject characteristics (quiescent state of target cells).[92] Of note, the promoters used in most current clinical trials have the lowest risk.[92] To date, over 60 patients with hemophilia have been treated with AAV liver-directed gene therapy with no reported significant safety or toxicity concerns,[8,10–14,22,24] however long-term follow up data are still being accumulated.

An alternative consideration for patients with HB and concomitant liver disease is the delivery of AAV to skeletal muscle.[9,102] Skeletal muscle gene therapy offers sufficient vascularity to allow secretion of synthesized FIX into the circulation and provides a way to overcome pre-existing NAbs. Canine studies demonstrated that intramuscular injection of cFIX in the UNC-CH HB dogs had an excellent safety profile,[103] but a similar injection protocol in the UAB HB inhibitor-prone dogs resulted in inhibitor formation.[104] These studies laid the foundation for a clinical trial of intramuscularly injected AAV2-FIX-WT,[9,102] which comprised the first *in vivo* parenteral gene therapy trial in humans. This study demonstrated safety and tolerability, albeit without significant increase in circulating plasma FIX activity.[9] However, all subjects demonstrated detectable FIX in muscle at biopsies collected at 2 and 10 months after gene therapy. In addition, two subjects demonstrated detectable FIX or vector in muscle biopsy samples obtained 3.7 and 10 years post intramuscular injection of the vector.[105,106] This study was limited to subjects with missense mutations based upon the HB canine experience. In preclinical studies, we demonstrated that the vector dose per site was the strongest predictor of immune response.[107] As the highest dose cohort in the human HB intramuscular trial required 80–90 injections[9] and did not result in circulating FIX activity, a different method of delivering FIX to the muscle was needed to facilitate higher vector doses. To this end, our group has demonstrated the ability to use isolated limb perfusion with transvenular delivery under hydrostatic pressure for skeletal muscle for gene therapy in HB dogs[108] with transient cyclophosphamide around vector infusion to prevent inhibitor development. Combining this delivery system with cFIX-Padua transgene, we recently demonstrated in both the UNC-CH and the UAB inhibitor-prone HB dogs not only therapeutic circulating FIX levels but also complete normalization of FIX for the first time,[109] without evidence of inhibitor formation despite multiple subsequent FIX-WT protein challenges.[109,110]

Overcoming pre-existing NAbs to AAV

As noted before, NAbs preclude effective viral transduction when delivered intravascularly.[8,111] Depending upon patient age, AAV serotype tested, and geographic region, between 30% and 70% of patients may have pre-existing NAbs to specific AAV serotypes.[19,33] The anti-AAV2

antibody prevalence seems highest but there is cross reactivity with other serotypes.[33] NAbs represent one of the most significant challenges to overcome in order to allow broad applicability of gene therapy to the hemophilia population at large. To date, studies have excluded patients with pre-existing NAbs to the study AAV serotype. Furthermore, differences in seroprevalence of immunoglobulin G (IgG) antibodies and neutralizing titer[112] suggest that non-NAbs may impact vector dose needed for efficacy.

Simple strategies to overcome the NAb titer include switching AAV serotype or trying to outcompete the antibody by increasing vector dose. Unfortunately, the cross reactivity of some NAbs limits the success of the first strategy. Other strategies to overcome NAbs have been studied, including adding empty capsids,[37] administration of immunosuppressive[34,35] drugs or plasmapheresis[36] to decrease titer, alteration of the AAV capsid,[113] and regional isolated AAV delivery to limit systemic exposure.[38] Unfortunately, all of these strategies are fraught with complications that make them suboptimal. Infusion of empty capsids allows for the adsorption of some of the circulating antibodies to allow transduction with transgene-encoded vector.[37] However, this strategy may be limited in those with high NAb titers and may increase the cellular immune response against the capsid as the total dose of vector would also need to be increased. Immunosuppression has been modestly effective at dampening but not eliminating the humoral response to AAV.[35] Further, B-cell depletion comes with associated lymphopenia and risk of infections. Plasmapheresis is a relatively safe and effective strategy for low-titer inhibitors ($\leq$1:20); however, despite five sessions plasmapheresis was not able to decrease NAb titers below 1:5 for patients with initially high titers (>1:20).[36] Patients with high-titer antibodies are more acutely in need of successful strategies and the limited efficacy of plasmapheresis is unlikely to be beneficial for this population. Localized liver perfusion[38] or isolated limb perfusion[108,114] with skeletal muscle delivery of vector, therefore, may be a viable option for patients with high NAb titers to liver tropic AAV serotypes. Likely, a combinatorial approach of these strategies will be needed to overcome pre-existing NAbs.

Lentiviral gene therapy

While AAV has emerged as the most successful vector for *in vivo* hemophilia gene therapy to date, there has been considerable effort in developing integrating vectors, such as retroviruses.[115] A potential advantage of this system is the ability to propagate the integrated transgene into progeny of the transduced cells, thereby avoiding potential dilution from organ or blood volume growth and allowing long-term therapy in young patients. Retroviruses are single-stranded RNA viruses containing a reverse transcriptase to allow for viral DNA production, which can then integrate into the host genome *via* an integrase. Initial studies demonstrated the ability to achieve therapeutic FIX (12–36%) and FVIII (116–139%) levels in neonatal hemophilic dogs treated with γ-retrovirus vector at 0.8–1.3 $\times$ 10^{10} transduction units (TU)/kg and 0.7–0.9 $\times$ 10^{10} TU/kg, respectively.[65,116] A phase I clinical trial sponsored by Chiron (San Diego, CA, USA) utilizing Moloney murine leukemia virus (MoMLV) carrying BDD-FVIII at doses of 2.8 $\times$ 10^{7} to 8.8 $\times$ 10^{8} TU/kg in patients with severe HA was shown to be safe and well tolerated.[117] However, there was a question of germline transmission of retrovirus *via* semen and FVIII activity was only transiently measured after infusion with no clear dose response. Furthermore, a clinical trial also using a retroviral vector in severe combined immunodeficiency (SCID) was complicated by leukemogenesis in four patients due to insertional mutagenesis,[118,119] which raised significant safety concerns.

More recently, studies have focused on lentiviruses, which are members of the retrovirus family but are different from simple MoMLV in that they are able to infect nondividing cells.[120] Of note, the oncogenesis seen in retroviral-mediated gene therapy for SCID defined above has not been seen in lentivirus-mediated gene therapy trials to date.[121] Lentiviral vectors are easily capable of carrying the *F8* cDNA and have decreased likelihood of preexisting NAbs in the population. Lentiviral transduction can occur either *ex vivo* with infusion of transduced autologous hematopoietic stem cells (HSCs) from the recipient or *in vivo via* intravenous infusion of vector targeting the liver. The *ex vivo* approach in HSCs can either be lineage restricted[122] or not.[123] In HA mice, transplantation of lentivirus-transduced HSCs with a human-porcine hybrid BDD-FVIII demonstrated therapeutic levels of 1–10%,[123,124] even in mice with pre-existing inhibitors. Delivery of platelet FVIII *via* transplantation of HSCs in a similar manner did result in disease amelioration in HA dogs,[122] but further studies are needed to understand if this is tolerogenic. *Ex vivo* HSC

lentivirus gene therapy is complicated by the need for conditioning regimens, bone marrow engraftment, or invasive procedures, which are likely to be significant hindrances for the subjects. Further large animal model studies are needed to understand the efficacy and safety profile of this approach. One approach that avoids the need for conditioning is the use of transduced autologous blood outgrowth endothelial cells (BOECs).[125] *Ex vivo* transduced autologous BOECs in three HA dogs *via* omental implantation with or without transient immunosuppression did initially result in therapeutic FVIII levels; however, this was not sustained due to the development of anti-FVIII IgG antibodies.[125]

Recently, *in vivo* intravascular delivery of lentiviral vectors carrying FVIII and FIX targeting the liver has been carried out in animal models.[126–128] The advantage of this vector-integrating system compared with AAV is the ability to allow long-term sustained transgene expression by avoiding potential transgene dilution when delivered to pediatric patients. In these studies, the lentivirus is modified through the addition of microRNA target sites to silence expression in nontarget cells. Recent studies have shown that the endogenous production of FVIII from the liver is mainly from liver sinusoidal endothelial cells rather than hepatocytes.[129,130] Building upon this observation, Merlin and colleagues treated HA mice with 1×10^9 TU of lentivirus containing BDD-FVIII under the control of an endothelial promoter and demonstrated therapeutic FVIII levels of around 3–8% with no inhibitor formation.[126] Even higher levels of FVIII activity were observed with further FVIII transgene optimization.[131] Furthermore, HA mice with inhibitors to FVIII treated with the same approach were able to eradicate pre-existing inhibitors and subsequently achieve expression of therapeutic FVIII levels. In HB, lentivirus expressing FIX-Padua modified to target the liver was successful in ameliorating disease phenotype in murine and canine HB models.[127,128] However, at doses of 5.7×10^8 to 2.3×10^9 TU/kg, FIX activity was only around 1% in the two dogs treated at the highest dose, despite use of cFIX-Padua in one dog.[128] Additionally, the first two dogs developed anaphylactoid infusion reactions necessitating steroid and antihistamine use in the third dog. Encouragingly, this vector was not associated with genotoxicity in a murine model[128] followed for around 12 months. However, the risks of insertional mutagenesis and genotoxicity in hemophilia subjects with a life expectancy over 60 years remains to be answered. Finally, *in vivo* lentivirus delivery is also complicated by vector stability,[132] immune response by antigen presenting cells to pseudotyped vector,[120] pre-existing antibodies to vesicular stomatitis virus used to pseudotype vector,[133] and complement binding to vector which can result in infusion reactions.[128] While lentivirus may offer significant advantages in hematopoietic and other stem-cell based disorders, there are significant challenges yet to overcome.

Gene-editing approaches

Gene-editing technologies are based on harnessing the natural repair machinery of the cell to modify DNA. Following a double-stranded break induced by a nuclease, repair progresses either through nonhomologous end joining (NHEJ) or homology-directed repair (HDR). NHEJ repair results in insertions or deletions (indels) of various lengths while HDR can be used to create precise site- and sequence-specific changes from a template.[134–136] Nucleases used to induce these breaks include zinc-finger nucleases (ZFN), transcription-activator-like effector nucleases and clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR associated protein 9 (CRISPR/Cas9).[137] Gene editing offers several advantages over AAV and integrating vectors, including precise editing, decreased insertional oncogenesis, and control under the endogenous promoter. While early animal studies utilizing ZFN and CRISPR/Cas9 did ameliorate disease phenotype in animal models,[138–140] more promising results were obtained by introducing the *F8*/*F9* genes into the albumin gene using 2A peptides by ZFN for targeting, resulting in complete and functional FVIII/FIX mRNA[141] under the control of the endogenous albumin promoter. A similar approach is now in clinical trials for HA and HB sponsored by Sangamo Therapeutics (Richmond, CA, USA) (Table 1) utilizing distinct AAV vectors to deliver the nuclease and two separate templates for the transgene (i.e. three different AAV vectors), but no data have been reported to date. Although gene editing holds significant promise for hemophilia, in its current state, the approach still requires AAV for delivery of the nuclease or transgene. Therefore, the same limitations for AAV gene therapy are present for gene-editing approaches and additional complications may arise as a result of the multitude of vectors needed. Moreover, limited data on off-target effects of these technologies prevents broader clinical application at this point.

Conclusion

Recent phase I/II AAV liver-directed gene therapy trials for hemophilia have shown remarkable decreases in ABR and factor usage, and engender optimism about the ability to significantly improve quality of life for persons with hemophilia. Lessons from early trials incorporating strategies for vector and transgene optimization and exclusion of subjects with pre-existing NAb to vector serotype have created the ability of a one-time infusion to provide simplified, lifelong prophylaxis which is a tremendous paradigm shift for patients and healthcare providers. Notably, there is convincing evidence that FIX-Padua may be the optimal transgene in HB gene therapy and there is considerable preclinical evidence that liver-directed gene therapy for ITI in HA has translational potential for a population with limited, suboptimal, and demanding therapeutic options otherwise. A one-time infusion that allows both tolerance induction and lifelong prophylaxis after inhibitor eradication with a single infusion would have immense benefits. The preclinical and clinical trial data have yielded a wealth of information regarding factors that permit success, but additional studies are needed to answer questions of optimal transgene, serotype, dose, target clotting factor activity, and manufacturing process as well as to understand the mechanistic processes underlying the immune response and rise in liver transaminases.

Currently, AAV gene therapy is limited in its general applicability due to pre-existing NAbs and pre-existent HCV or HBV infection. Long-term follow-up studies will be critical to assessing and addressing the safety concerns and therefore allow enrollment of younger patients, as further evidence of safety around risk of genotoxicity is amassed and ways to overcome NAbs for potential reinfusion are established. In the coming years, as some trials move beyond phase I/II, we anticipate the recognition of promising strategies that would yield benefits for many more patients. Further, these studies will likely help standardize aspects such as NAb titers, manufacturing process, and serotype. Finally, there are considerations of the cost of gene therapy, both to patients and payers. Arguably, the most striking benefit would be in the developing world, where routine access to clotting factors is limited, but how gene therapy is priced will affect its availability to these patients. There is a moral imperative to develop economically priced strategies to improve the burden of disease for patients in the developing world.

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Conflict of interest statement

The authors declare that they have no conflicts of interest.

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