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Expert Opinion on Biological Therapy

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iebt20

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To cite this article: Steven W. Pipe, Gil Gonen-Yaacovi & Oscar G. Segurado (2022): Hemophilia A gene therapy: current and next-generation approaches, Expert Opinion on Biological Therapy, DOI: 10.1080/14712598.2022.2002842

To link to this article: https://doi.org/10.1080/14712598.2022.2002842

Published online: 06 Jan 2022.

EXPERT OPINION ON BIOLOGICAL THERAPY
https://doi.org/10.1080/14712598.2022.2002842

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REVIEW

Hemophilia A gene therapy: current and next-generation approaches

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ABSTRACT

Introduction: Hemophilia comprises a group of X-linked hemorrhagic disorders that result from a deficiency of coagulation factors. The disorder affects mainly males and leads to chronic pain, joint deformity, reduced mobility, and increased mortality. Current therapies require frequent administration of replacement clotting factors, but the emergence of alloantibodies (inhibitors) diminishes their efficacy. New therapies are being developed to produce the deficient clotting factors and prevent the emergence of inhibitors.

Areas Covered: This article provides an update on the characteristics and disease pathophysiology of hemophilia A, as well as current treatments, with a special focus on ongoing clinical trials related to gene replacement therapies.

Expert Opinion: Gene replacement therapies provide safe, durable, and stable transgene expression while avoiding the challenges of clotting factor replacement therapies in patients with hemophilia. Improving the specificity of the viral construct and decreasing the therapeutic dose are critical toward minimizing cellular stress, induction of the unfolded protein response, and the resulting loss of protein production in liver cells. Next-generation gene therapies incorporating chimeric DNA sequences in the transgene can increase clotting factor synthesis and secretion, and advance the efficacy, safety, and durability of gene replacement therapy for hemophilia A as well as other blood clotting disorders.

ARTICLE HISTORY
Received 3 August 2021
Accepted 2 November 2021

KEYWORDS
Adeno-associated virus; cellular stress; clinical trials; coagulation; factor viii; gene therapy; hemophilia A; inhibitors; transgene; unfolded protein response

1. Introduction

1.1. Hemophilia, a group of inherited bleeding disorders

Hemophilia is classified as a group of X-linked inherited hemorrhagic disorders resulting from the absence of or defects in critical factors in the coagulation cascade [1]. Patients with hemophilia exhibit compromised thrombin generation and fibrin clot formation, which leads to bleeding episodes, most commonly into the joints (hemarthrosis). The 2 main types of hemophilia (A and B) relate to deficiency or dysfunction of the specific clotting factors VIII or IX, respectively, with the severity depending on the level of clotting factor activity. Hemophilia affects mainly males, and hemophilia A accounts for the large majority of cases (~80%), affecting approximately 1 of every 5000 live-born males. Hemophilia B is 5 times less common than hemophilia A, having an incidence of approximately 1 in 30,000 births [2,3]. Over a million people around the world are estimated to have hemophilia, including more than 30,000 in the United States (US) [4]. The prevalence is higher among Whites than Blacks or Hispanics [5].

A meta-analysis of national registries from 6 countries, including Australia and the United Kingdom [6], revealed that the prevalence of hemophilia A is 17.1 cases per 100,000 males when including all severities and 6.0 cases per 100,000 males when including only severe hemophilia A. Although these estimates are higher than previously reported, the prevalence still characterizes hemophilia as a rare disease according to definitions used in the US (<200,000 cases) and the European Union (<5 cases/10,000 persons) [6].

1.2. Hemophilia A – disease characteristics and manifestations

The most common of the 2 main types of hemophilia is hemophilia A, which is caused by decreased activity of plasma coagulation factor VIII (FVIII) due to mutations of the *F8* gene encoding this protein. The severity of hemorrhagic episodes tends to correlate directly with the plasma FVIII concentration, with 5% to 40% of normal considered mild, 1% to 5% of normal considered moderate, and less than 1% of normal considered severe [1,7]. In mild hemophilia, bleeding is more likely to occur following trauma or surgery, and unprovoked hemorrhages are rare. In moderate hemophilia, bleeding is usually observed after injuries, but spontaneous bleeding episodes with no obvious cause may also occur. In severe hemophilia, patients experience recurrent spontaneous bleeding events with hemarthroses, bleeding into the muscles and soft tissues, and other life-threatening bleeds (e.g. intracranial hemorrhage), as well as excessive bleeding during and following surgery or trauma. Recurrent hemarthrosis leads to hypertrophic synovitis, progressive cartilage degradation, and hemophilic arthropathy characterized by chronic pain, severe deformity, and reduced mobility [1,7–10].

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Article highlights

- Hemophilia is a rare monogenic disorder and an ideal candidate for gene therapy.
- Several adeno-associated virus (AAV)-based gene therapies for hemophilia A are currently under evaluation in clinical studies.
- More efficient transgene vectors are needed to provide sufficient and sustained levels of coagulation factor VIII (FVIII).
- The packaging capacity of AAV vectors limits the size of the inserted vector genome.
- A human/porcine chimeric second-generation factor VIII gene therapy having the smallest recombinant AAV2/8 vector compared with other clinically tested gene therapy constructs has been developed.
- The chimeric transgene encodes a liver-specific, codon-optimized bioengineered B-domain–deleted FVIII with a hepatocyte-specific promoter exhibiting more than 10-fold increased biosynthesis of FVIII compared with standard human F8 transgenes.
- Pre-clinical data indicate that the chimeric transgene is associated with a lower unfolded protein response in the endoplasmic reticulum, which may account for its enhanced biosynthetic efficiency and potential extended durability.

1.3. Hemophilia A – disease burden

Hemophilia A is a chronic life-threatening condition that confers a considerable clinical, psychologic, and economic burden on patients and caregivers, thereby affecting their quality of life [11–15]. Currently recommended therapy for hemophilia-related bleeding episodes in patients with severe disease is prophylaxis with clotting factor replacement products administered intravenously 2 or 3 times per week [16]. About 30% of the patients develop anti-FVIII neutralizing alloantibodies (inhibitors), however, which is a serious complication of this treatment [17]. Patients with hemophilia that develop inhibitors have impaired health-related quality of life, greater clinical burden, and higher resource utilization compared with patients who do not develop inhibitors, as well as a higher number of annual bleeds, joint bleeds, pain, and hospitalizations [18,19]. Further, the high frequency of the treatment regimen creates a significant burden for the patient, caregiver, and healthcare system.

Patients with non-severe hemophilia also suffer considerable morbidity and an increased mortality risk [20,21]. Even in patients with mild hemophilia, the mean number of bleeding episodes is 0.44 to 4.5 per year, which severely interferes with their quality of life [22]. Males with hemophilia have a lower life expectancy than the general male population, even after treatment-related improvements [23–25]. The decrease in the life expectancy of patients with hemophilia A in developed countries is 30%, and for those with severe hemophilia A, 37% [6].

1.4. Hemophilia A – disease pathophysiology

Hemophilia A is caused by an undersupply of the essential blood clotting factor FVIII, encoded by the *F8* gene located on the X chromosome. The bleeding severity depends on the plasma FVIII levels, which vary according to the specific mutation. The *F8* gene is a large gene comprising 26 exons. In severe hemophilia A, FVIII activity is almost completely abolished, which is most commonly (~45%) caused by a large intron 22 inversion of the *F8* gene [26]. Point mutations causing hemophilia comprise 85% missense mutations which can lead to quantitative or qualitative alteration of protein biosynthesis, secretion, activity or clearance. In some cases, the exonic changes may have detrimental effects on mRNA splicing. Another 15% are nonsense mutations, and a small percentage (5%) of large or small deletions and insertions as well as inversions within intron 1 [27,28].

FVIII is synthesized by hepatic and extrahepatic sources, likely of endothelial origin. Extrahepatic sources include Kupffer cells, monocytes, and monocyte-derived macrophages within the hematopoietic system. The liver is a major source, and while hepatocytes are the most abundant cell type comprising the liver, the liver sinusoid endothelial cells are the main source of liver-derived FVIII [29–31]. Upon its release into the circulation, the FVIII heterodimer forms a tight noncovalent complex with von Willebrand factor (vWF), the FVIII carrier protein produced and secreted by vascular endothelial cells. The half-life of FVIII in the absence of VWF is only 2 h compared to 12 h when bound to VWF [32]. The 2332-amino acid FVIII protein has 6 domains (A1-A2-B-A3-C1-C2), circulating as a 90-200 kDa heavy chain (A1-A2-B) and an 80-kDa light chain (A3-C1-C2). When the coagulation cascade is triggered by the presence of thrombin and activated factor X, vWF dissociates from FVIII and serine proteases act to cleave FVIII with release of the B domain, which has a potential regulatory role [27]. The activated form of FVIII (FVIIIa) functions as a cofactor for factor IXa within the factor X-activating complex and accelerates the proteolytic conversion of factor X to its activated form (Xa) in the presence of calcium ions and phospholipids [33–36]. High FVIII activity is associated with an increased risk of stroke, and low levels adversely affect bone metabolism [37].

1.5. Hemophilia A – outcome measures

The main goal of therapy is to decrease the number of bleeding episodes that patients with Hemophilia A experience. Consequently, annualized bleeding rate (ABR) has become the primary outcome measure in studies of hemophilia therapies [38]. ABR, however, is essentially a patient-reported outcome in which the patient records the occurrence of bleeding events, their location, severity, and whether there was a precipitating event. Due to the subjective nature of this measurement, clotting factor activity, which is considered a more accurate and objective primary endpoint, has been proposed as a more objective measure of assessing therapeutic efficacy [39]. FVIII activity levels (endogenous as well as plasma levels achieved through prophylaxis) have long been used to define the phenotypic risk of bleeding and progression of joint complications across the range of disease severity [39]. The advent of bioengineered FVIII therapeutics (**see Sect. 1.6**), however, has introduced discrepancies in the in vitro FVIII activation profile that are observed when these molecules are measured by the traditional one-stage clotting assay (activated partial thromboplastin time) as compared to a chromogenic assay [40]. This discrepancy has also been observed within gene therapy clinical trial programs, with the one-stage assay reporting ~1.6-fold higher FVIII activity compared to the

chromogenic assay [41]. Accordingly, this has created uncertainty within the context of gene therapy regarding the correlation of bleed protection with the level of expressed FVIII across all ranges observed depending on whether the one-stage or chromogenic assay is used. Therefore, both FVIII activity (by either assay method or both) and ABR have become primary outcome measures.

1.6. Hemophilia A – current and future treatment options

The current standard of care for hemophilia A is prophylaxis aimed at increasing FVIII levels to a sufficient level to prevent bleeding episodes and reduce the occurrence of hemarthrosis and subsequent joint disease by regular intravenous infusion of exogenously derived FVIII concentrates [42–45]. Maintaining FVIII trough levels at 3% to 5% is desirable to prevent bleeding events, as even occasional clinical and subclinical bleeding episodes can lead to the progression of joint disease over the patient's lifespan. To provide effective bleed prophylaxis in hemophilia A, frequent intravenous infusions of FVIII concentrates (every 2–3 days) are required because of the relatively short half-life of FVIII in the circulation. The use of exogenous FVIII concentrates generally provides an ABR of 2 to 5 [46], but the burden of frequent administration and the difficulty obtaining and maintaining therapeutic FVIII levels have prompted the development of therapies with longer half-lives.

Modified recombinant products with enhanced pharmacokinetic properties (e.g. recombinant FVIII-Fc fusion protein or conjugation with polyethylene glycol) allow for less frequent infusions (every 3–5 days, or even once weekly in some patients) [47] and the ability to target higher trough levels. These strategies, however, are limited to a 1.3- to 1.5-fold half-life extension of FVIII because clearance of the recombinant protein from the blood is largely regulated by its interaction with vWF [48–51]. The various extended half-life recombinant FVIII products have improved the ABR, with values ranging from 1.2 to 1.9 and pivotal clinical studies showing a progressive decrease in ABR during extension phases [52]. These new products are gaining popularity, and in 2019, approximately 28% of individuals with severe hemophilia A in the US received extended half-life factor products, 7.1% were prescribed non-factor products, and a decreasing proportion of patients (64.0%) continued to be treated with standard half-life factor products [53]. Unfortunately, factor replacement therapy is associated with the development of inhibitors (alloantibodies) to the infused concentrate that neutralize FVIII activity and render FVIII replacement therapy ineffective [54,55]. The development of inhibitors occurs in approximately 30% of patients with severe hemophilia A and 13% of patients with non-severe hemophilia [56–58]. The pathophysiology of inhibitor development is thought to involve both genetic and environmental factors [59,60]. A large F8 gene deletion that leads to little or no FVIII production is the strongest predictor of FVIII immunogenicity, and is also more likely to be associated with inhibitor development compared to missense mutations [61].

One therapeutic option for managing bleeding episodes in patients who develop inhibitors is to administer bypassing agents (e.g. recombinant activated factor VII or activated prothrombin complex concentrate containing activated serine proteases) [61–63]. A novel bispecific antibody (emicizumab) was recently licensed in both the US and Europe for the prevention of bleeding in all patients with hemophilia A, regardless of the presence or absence of inhibitors [64,65]. Emicizumab recognizes both activated factor IX (FIX) and factor X, and mimics FVIIIa cofactor activity. Owing to its longer half-life and subcutaneous administration, emicizumab has significantly improved the treatment of hemophilia A, regardless of the presence of FVIII inhibitors [66–68]. Other nonfactor therapies are in development, such as fitusiran (RNA interference therapy administered subcutaneously once monthly) and monoclonal anti-tissue factor pathway inhibitor antibodies, such as concizumab and marstacimab [69–74]. These investigational agents also offer the advantages of subcutaneous delivery and up to monthly or every other month dosing. An alternative approach to dealing with inhibitors is to attempt to eradicate them.

Currently, immune tolerance induction (ITI) is the only established therapy for abolishing FVIII inhibitors and achieving tolerance to FVIII. ITI utilizes repeated dosing regimens of FVIII (40–300 IU/kg) at 1- to 3-day intervals [61]. The success rates of the current ITI protocols range from 60% to 80% with the pre-ITI anti-FVIII titers correlating with the prognosis, but the success rates range widely according to several factors, including age at ITI start, race/ethnicity, FVIII genotype, and historical inhibitor peaks [75]. ITI is costly and the compliance burden is challenging for both the patient and caregiver [61]. Treatment with emicizumab to mimic activated FVIII may be an option for patients who have developed inhibitors and are not candidates for ITI, and studies of its use alone or in combination with FVIII in ITI are in progress [76]. Alternative investigational strategies for ITI to FVIII are based on the development of new technologies, including gene therapy, regulatory T-cell therapy, and transgenic plants for inducing oral tolerance [77].

2. Gene therapy for hemophilia

2.1. Gene therapy overview

The modification and transfer of genetic material to compensate for abnormally mutated genes is referred to as gene therapy. The aim of gene therapy is to treat or even prevent genetic diseases by inducing long-term expression of the transferred gene at therapeutic levels [78,79]. Hemophilia is a hereditary disorder whose genetics are well understood, making it an ideal target for gene therapy. Further, because the severity of the bleeding phenotype is relatively insensitive to the plasma levels of the coagulation factors, precise regulation is not necessary. The greatest limitation of the currently available treatments is related to their short therapeutic half-life, resulting in frequent intravenous infusions and leading to intense efforts to develop more effective gene therapy strategies [71,80–85].

Two types of vectors are most often used for current gene therapy strategies. Lentiviral vectors are used for ex vivo gene transfer into hematopoietic and other stem cells [83,86], and adeno-associated viral (AAV) vectors are generally used for in vivo gene transfer into postmitotic cells [78,87]. Because lentiviral vectors are very challenging to manufacture, clinical studies using these vectors have not yet been initiated due to the difficulty in producing the amount of vector required for in vivo delivery [39,88]. Wild-type (wt) AAV is a small single-stranded DNA virus of the Parvovirus family that is nonpathogenic and replication-defective such that it cannot induce disease. Current clinical studies of gene therapies for hemophilia use recombinant AAV (rAAV) vectors for direct transduction of coagulation factor genes into liver hepatocytes, which are converted to protein biofactories that produce and secrete the transgene product into the circulation. Once the target cell is transduced, the rAAV therapeutic gene sequences are found mostly as concatemeric episomes with a low level of integration into the genomic DNA of the host [87,89].

AAV has a wide range of naturally existing serotypes, each having distinct organ/cell tropism [90]. Hybrid serotypes can also be manufactured to increase the vector efficiency. The initial clinical studies in hemophilia used the first-generation AAV serotype, AAV2, which is the best characterized and most extensively studied of the serotypes. Additional serotypes that have been tested are AAV5, AAV8, and AAVrh10 [91]. Serotype AAV8 efficiently transduces genes to the liver and promotes high gene expression even when injected intravenously [87,92]. AAV5 is the most phylogenetically distinct vector serotype in terms of the capsid structure, whereas the other commonly used serotypes share over 80% homology [91].

One limitation of AAV vectors is that they have limited packaging capacity (~4.7 kilobases [kb]) [93]. Thus, the initial gene therapy studies for hemophilia were conducted in hemophilia B using the smaller *F9* transgene [94]. Similar clinical studies were slow to start for hemophilia A because the *F8* transgene is ~7 kb and *F8* has a poor expression profile [95,96]. An AAV-based gene-transfer approach to address the size constraints was recently developed by removing the FVIII B-domain (referred to as B-domain deleted [BDD]) to reduce the size of the FVIII expression cassette [96]. In addition, the relatively poor FVIII expression profile can be improved 10-fold by codon optimization (i.e. engineering the codon to improve gene expression and protein translation on the basis of the host codon bias) of human FVIII wt cDNA [97]. In 2017, BioMarin Pharmaceutical successfully applied this construct using a codon-optimized AAV5 vector encoding a BDD human FVIII vector (AAV5-hFVIII-SQ) [98]. For a review of clinical studies of gene therapy in hemophilia A, refer to Section 3.

2.2. Limitations and risks associated with gene therapy in hemophilia

Gene therapy targeting hemophilia involves intravenous administration of the *F8* transgene within a viral capsid (Figure 1). Intravenous administration leads to preferential targeting of the transgene to the hepatocyte because of the architecture of the liver's capillaries [99]. Once the host cell identifies the AAV capsid through its glycosylated cell surface receptors, the virus is internalized via clathrin-mediated endocytosis and transported in the cytosol via the cytoskeletal network. The AAV must escape from the endosome at the optimal time to avoid lysosomal degradation and to promote its transport to the nucleus and its subsequent uncoating through the conformational changes of pH-sensitive endosomolytic viral proteins [100]. The viral inverted terminal repeats present in the rAAV genome drive either intramolecular or intermolecular recombination (i.e. concatemerization) to form circularized genomes that survive as episomes in the nucleus [101].

A major limitation of the AAV-based gene therapy approach is that episomal AAV genomes are not replicated during cell division. Important points to consider when using this approach are the potential loss of factor expression and the consequences of liver growth and dilution of transduced hepatocytes in younger patients [71]. Unfortunately, repeat administration is contraindicated because after the first dose, a humoral immune response is generated against the AAV

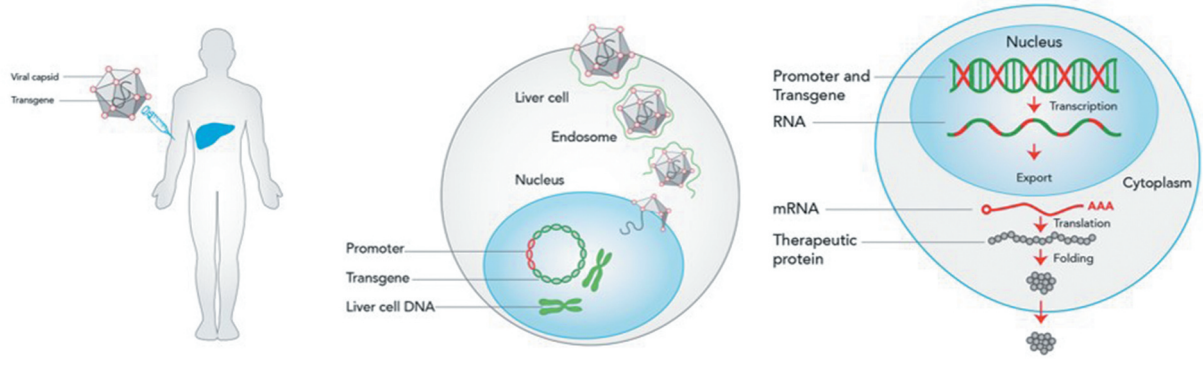

Figure 1. **Targeted gene therapy schematic**. The transgene of interest is packaged inside a recombinant viral vector and injected into the subject. The vector is taken up by many different cell types via endocytosis into organelles called endosomes. The vector escapes from the endosomes, attaches to the nuclear envelope, and injects its genomic payload into the nucleus. The vector genome contains a tissue-specific promoter such that it is only transcribed in the target cell type (e.g. hepatocytes). The host transcription machinery transcribes the transgene into mRNA, which is transported out of the nucleus and translated into the protein of interest.

capsid proteins. It is possible, however, for the rAAV vector to integrate into animal genomes [102], which might alleviate the dilution effect. It is important to consider the potential for the development of genotoxicity, which, although rare, can occur [103].

Key challenges of gene therapy in general include large-scale vector manufacture and cost, quality control, and assay standardization, as well as immunologic barriers to rAAV gene delivery. Difficulties removing cellular and viral impurities from rAAV particles as well as empty AAV capsids, lack of standardization, and inherent batch-to-batch variations in vector potency affect production costs [101]. The main limitations and risks of gene therapy in hemophilia are detailed below.

2.2.1. Anti-AAV neutralizing antibodies

Preexisting neutralizing anti-AAV antibodies specific to the various AAV serotypes (with varying degrees of serologic cross-reactivity) that can neutralize the vector and thereby reduce treatment efficacy are prevalent in the population due to natural infection with wt AAV during childhood [92,100,104–106]. The prevalence of anti-AAV antibodies varies from 20% to 70%, depending on the specific AAV serotype and subject population [39,87,91,107]. A smaller subject population exhibits preexisting antibodies to AAV5, which is probably the result of lower cross-reactivity with primate AAV antibodies [92]. Assessment of the prevalence of preexisting immunity to AAV in the general population is complicated, however, by the fact that laboratory tests for detecting anti-AAV antibodies are not yet standardized [39,87,107,108].

In addition to preexisting immunity, a delayed cellular immune response to the AAV capsid, typically occurring 4 to 12 weeks after vector infusion, may result in destruction of the transduced cells by cytotoxic T lymphocytes and a loss of therapeutic efficacy (i.e. decreased durability over time). The induction of cytotoxic T lymphocytes prevents effective repeat dosing and attenuates the long-term therapeutic benefits [61,79,109]. In addition to the capsid protein, other AAV vector components, such as stimulatory hypomethylated CpG motifs, can impact the immune response [61,110–113].

Some therapeutics based on AAV2, AAV8, or other similar serotype vectors produced in mammalian cells induce an anti-AAV capsid cellular immune response that targets transduced hepatocytes, resulting in the loss of transgene expression, which can sometimes be controlled by immunosuppressive therapy [114]. Ongoing studies may determine whether such cellular immune responses can be avoided by producing the AAV vector in more distantly related species, such as insects. Some studies demonstrated that preexisting neutralizing AAV antibodies can interfere with vector transduction and limit therapeutic efficacy (see Section 2.2.3) [115], while in others, transgene loss or evidence of cellular immunity have not been reported [116]. In one clinical trial program for hemophilia B, the transduction efficiency did not appear to be affected by preexisting antibodies to AAV5 at titers commonly observed in the general population, and the clinical trial currently in progress has not excluded subjects with preexisting anti-AAV capsid neutralizing antibodies [91,115,117,118].

The impact of neutralizing antibodies may be overcome by strategies such as changing the AAV serotype or increasing the vector dose to outcompete the antibody. Changing the AAV serotype, however, may not be effective due to the cross reactivity of some neutralizing antibodies. Other potential strategies include incorporating empty capsids, decreasing titers with immunosuppressive drugs or plasmapheresis, altering the AAV capsid, and isolating AAV delivery to a limited area to reduce systemic exposure. All of these strategies, however, compromise the expected efficacy of the treatment [119].

Another approach is to attempt to prevent the development of adaptive immunity and T cell immune responses to the AAV capsid. For example, tolerogenic nanoparticles encapsulating rapamycin were recently developed to induce immune tolerance and thereby enhance transgene expression after the first dose of an AAV vector and inhibit adaptive antibody and T cell immune responses against the AAV capsid, allowing for repeated administration of AAV vectors in mice and nonhuman primates [120,121].

Most present-day hemophilia gene therapy studies target patients that are negative for preexisting anti-AAV capsid neutralizing antibodies. Thus, these antibodies prevent the currently available gene therapies from being widely applied to the hemophilia population. In addition, because AAV neutralizing antibodies can develop following the administration of AAV-based gene therapy, patients cannot be treated with multiple doses of the same AAV serotype [87].

2.2.2. Transgene product inhibitors

To date, the emergence of FVIII or FIX inhibitors in clinical AAV gene therapy studies has not been reported. The *F8* and *F9* genotypes of these participants have not yet been reported. In general, however, current clinical studies only include patients who have had >150 exposure days to factor replacement and anyone with a history of inhibitors was excluded. Thus, the participants in these studies are likely enriched with *F8* and *F9* genotypes associated with a low risk for inhibitor formation. It is not known if treatment-naïve patients can form inhibitors following gene therapy [84,95]. Thus, clinical trials are in progress to evaluate individuals with active inhibitors to determine if gene therapy can induce tolerance and eradicate inhibitors [92].

2.2.3. Hepatotoxicity

Clinical studies of both hemophilia A and B report asymptomatic transient increases in alanine transaminase (ALT) levels that can be controlled with a tapering course of glucocorticoids [98,122,123]. This typically mild toxicity may be related to viral particle trafficking, uncoating, and the DNA damage response induced by the vector DNA [98]. A few studies have shown that the increase in ALT levels coincides with a detectable anti-AAV capsid T-cell response, but the findings have not been consistent. In some patients, ALT levels are increased without a capsid response [124], whereas in other patients a capsid response is observed without a concurrent increase in the ALT levels [94,125]. The increase in ALT levels observed following AAV-based gene therapy is dependent on vector dose, but is not associated with the AAV capsid, genome

configuration, transgene promoter, or manufacture method [96]. In the BioMarin FVIII gene therapy Phase 1/2 study (Section 3.2.1), an increase in ALT was not generally associated with lost FVIII activity or a T-cell immune response to viral capsid peptides [92,126].

Most ALT elevations, however, reach a maximum level 1.5- to 2-fold higher than the upper normal limit and may or may not be associated with hepatocyte loss [92]. The US National Hemophilia Foundation's Medical and Scientific Advisory Committee recommends obtaining biopsies to determine hepatocyte death or damage, T-cell cytotoxicity, innate immune response and inflammation, FVIII/FIX expression and distribution, and evidence of residual intracellular AAV capsids in at least a subset of clinical study subjects to address the safety, efficacy, durability, and variability of the response [127,128]. These endpoints critically contribute to our understanding of the long-term safety and efficacy of the treatment, as well as hepatocyte turnover, and may facilitate the detection of differences in the tropism of AAV serotypes [92].

2.2.4. Tumorigenesis

Proviral DNA is usually maintained in episomes of the transduced cell nuclei. Thus, the risk of genomic insertional mutagenesis after AAV-mediated gene transfer is low, consistent with the fact that although it is common for humans to be infected with wt AAV, AAV infection is not associated with oncogenesis [96]. As rare as the integration of the AAV genome in the host is, however, deep sequencing studies have demonstrated that such integration does take place in the liver [129,130], and several recent studies revealed a link between hepatocellular carcinoma and wt AAV [131–133]. Although substantially more evidence supports the lack of a risk for insertional mutagenesis in both animal models and AAV-treated patients with hemophilia, ruling out this potential risk will require further studies with a larger number of treated patients [84]

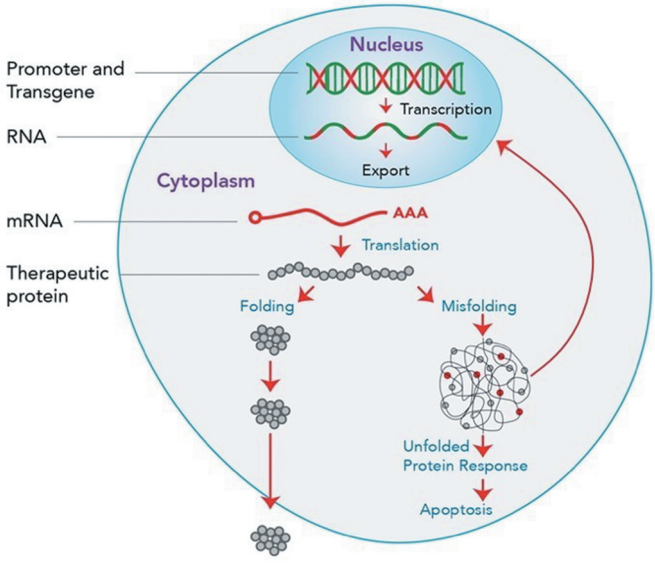

Figure 2. **Schematic diagram of the unfolded protein response (UPR).**

2.2.5. Cellular stress

The number of synthetic and other complex biologic functions carried out by the liver makes it highly susceptible to endoplasmic reticulum (ER) stress [134,135]. Because transgene expression is limited to a subset of cells, some individual cells may produce an overload of FVIII, which can induce cellular stress. The ER is where nascent proteins are folded and secreted. ER function overload, such as that induced by a greater demand for protein folding or the accumulation of unfolded or misfolded proteins, leads to the unfolded protein response (UPR) [136], an indication of the cellular stress response [137,138] (Figure 2).

Cellular stress is induced when cells produce too much protein or the proteins are not processed correctly and are unfolded or misfolded. This cellular stress engages the UPR to produce more endoplasmic reticulum to ameliorate the unfolded and misfolded proteins. If the cellular stress is too great, the UPR can lead to cell death via apoptosis. The UPR activates downstream signaling cascades by upregulating genes in the nucleus that lead to translational arrest and protein degradation that reduce the protein load in the ER [139].

The UPR is a coordinated cellular mechanism that regulates protein synthesis and secretion in the ER [140]. It functions as an adaptive signaling pathway that prevents misfolded and unfolded proteins from accumulating in the ER, thereby minimizing oxidative stress [141,142]. The UPR comprises 3 transmembrane ER stress sensor proteins, including inositol-requiring kinase, activating transcription factor 6, and protein kinase activated by double-stranded RNA-like ER kinase [143,144]. UPR induction can be measured by assessing ER response element-luciferase reporter activity, X-box-binding protein 1 splicing, and upregulation of immunoglobulin-binding protein (BiP), also called Grp78 [145]. Grp78/BiP is a central regulator for ER stress due to its role as a major ER chaperone with anti-apoptotic properties as well as its ability to control the activation of transmembrane ER stress sensors through a binding-release mechanism [141,146–148]. Chronic activation of the UPR and the accumulation of unfolded proteins in the ER can result in cell death via apoptosis. Although hepatocytes are targeted by gene therapies to produce FVIII protein, they do not naturally express either FVIII or vWF [31], which increases the risk of inducing ER stress by overexpressing FVIII in these cells [84]. Overexpressed FVIII is prone to misfolding in the ER lumen, which activates the UPR, leading to cell damage or apoptosis. An increase in the UPR correlates with reduced FVIII expression as assessed with in vitro cellular expression systems, as well as with reduced plasma FVIII concentrations in vivo following gene transduction by viral gene therapy vectors [40]. Interestingly, the biosynthesis of a porcine FVIII construct containing the A1 and ap-A3 domains is 10 to 100 times more efficient than that of human FVIII, and confers a higher level of expression and secretion efficiency [149,150]. Expression of human FVIII activates the UPR to a greater extent than does expression of porcine FVIII [145].

2.2.6. In-vivo gene editing

Targeted genome-editing techniques to correct gene mutations at the genome level using programmable nucleases (e.g.

zinc-finger nuclease, transcription activator-like effector nuclease, and clustered regularly interspaced short palindromic repeat [CRISPR]/CRISPR-associated protein 9 [Cas9] systems [151]) may provide a more enduring treatment for hemophilia [102,152,153]. A zinc finger nuclease (ZFN)-based gene editing study was first explored in hemophilia B patients whereby it placed a normal *F9* transgene within the albumin intron 1 under control of the endogeous albumin locus promoter. However, the program was terminated (NCT02695160). Recent findings showed that in vivo genome targeting of the human transgene into the Alb locus by CRISPR/Cas9 led to human FVIII production in the liver and ameliorated severe hemophilia A phenotype in mice [153]. Such genetic approaches effectively translated to humans may provide more permanent solutions to patients with hemophilia A.

2.3. Summary

To date, the most serious concern associated with liver-targeted delivery of AAV is liver toxicity accompanied by a loss or reduction in transgene expression [96]. Both the vector dose and optimal transgene expression may affect whether an immune response is induced against the AAV vector and transgene product [84,154,155]. The precise pathophysiologic mechanism for the loss of transgene expression and hepatotoxicity, however, is unclear [96]. The underlying mechanisms are likely complex and may involve other factors, such as the UPR [95]. Although there is evidence for the existence of memory CD8 + T cells targeting AAV capsids, the response is not yet completely clear, and loss of transgene expression or hepatotoxicity does not always correlate with a T-cell response. Therefore, the ability to generate durable, long-term expression of exogenous genes will require a more complete understanding of both innate and adaptive immune responses to AAV vectors, and further elucidation of other sources of cellular stress and toxicity [61,84,156].

Children are currently not eligible for gene therapy targeting hemophilia. As the FVIII and FIX expression cassettes are in episomes and therefore not replicated during cell division, treating a patient whose liver is still developing may lead to diluted expression [84]. A study in adults revealed stable expression of the transgene for at least 10 years for FIX, but a 50% decrease in FVIII expression between years 1 and 2, which continued to decrease until the end of year 3 [92].

Long-term goals for investigational gene therapy in hemophilia include increasing the durability of transgene expression and the ability to treat patients with preexisting anti-AAV neutralizing antibodies or FVIII and FIX inhibitors [61,78,96]. A chimeric human/porcine FVIII molecule (ET3) with enhanced secretory capacity is a potential solution for obtaining stable and durable transgene expression [149,157]. Further, it is necessary to collect long-term data on the safety, variability, and durability of efficacy [158].

3. Current clinical studies of gene therapy for hemophilia A

3.1. Timeline of gene therapy clinical trials for hemophilia A

Table 1 presents the chronologic flow of active gene therapy studies for hemophilia A per the timeline reported at ClinicalTrials.gov (sorted by study start date). Overall, 16 clinical studies of gene therapy for hemophilia A have been registered at ClinicalTrials.gov.

3.2. Summary of gene therapy clinical trials for hemophilia A

Gene therapies for hemophilia A developed by 8 companies are currently being evaluated in clinical studies, as summarized in Table 2 and the ensuing sections.

3.2.1. Biomarin Pharmaceutical

BioMarin Pharmaceutical is conducting 5 clinical studies to evaluate the effects of various gene therapy regimens for severe hemophilia A utilizing AAV5–hFVIII-SQ (valoctocogene roxaparvovec, Roctavian), which contains the 14-amino-acid human-derived SQ linker sequence in place of the B domain

Table 1. Timeline of gene therapy clinical studies in Hemophilia A.

Company/product	Study Phase	ClinicalTrial.gov Identifier Number	Study Start Date	Estimated Primary Completiona	Estimated Study Completion Date
BioMarin/Roctavian	Phase 1/2	NCT02576795	Aug 2015	Mar 2024	Mar 2024
Spark/ SPK-8011	Phase 1	NCT03003533	Jan 2017	May 2020	May 2020
UCL/AAV2/8-HLP-FVIII–V3	Phase 1	NCT03001830	Jun 2017	Dec 2020	Jun 2025
Pfizer-Sangamo/SB-525	Phase 1/2	NCT03061201	Jun 2017	Jul 2024	Jul 2024
BioMarin/Roctavian	Phase 3	NCT03370913	Dec 2017	Dec 2022	Sep 2023
BioMarin/Roctavian	Phase 3	NCT03392974	Mar 2018	Dec 2022	Mar 2024
Takeda-Shire/ TAK-754	Phase 1/2	NCT03370172	Mar 2018	Sep 2021	Sep 2024
BioMarin/Roctavian	Phase 1/2	NCT03520712	Apr 2018	Jun 2025	Jun 2025
Pfizer-Sangamo/SB-525	Phase 3	NCT03587116	Jul 2018	Sep 2021	Sep 2021
Spark/ SPK-8011	Observational	NCT03432520	Aug 2018	Dec 2022	Dec 2022
Bayer-Ultragenix/BAY 2599023	Phase 1/2	NCT03588299	Nov 2018	May 2022	Jul 2026
Spark/SPK-8016	Phase 1/2	NCT03734588	Jan 2019	May 2020	May 2020
BioMarin/Roctavian	Phase 3b	NCT04323098	Jun 2020	Dec 2021	Dec 2025
Shenzhen/YUVA-GT-F801	Phase 1	NCT03217032	Jun 2020	May 2022	Jun 2022
Pfizer-Sangamo/SB-525	Phase 3	NCT04370054	Jul 2020	Aug 2022	Nov 2026
Expression Therapeutics	Phase 1	NCT04418414	Feb 2021	Apr 2025	Apr 2039

aPrimary completion date is the date on which the last subject was examined or received an intervention to collect final data for the primary outcome measure.
Source: https://www.clinicaltrials.gov (date accessed: 20 April 2021)

Table 2. Overview of gene therapy clinical studies for Hemophilia a by company.

Product	Study number/title (**ClinicalTrial.gov identifier number**)	Status
BioMarin Pharmaceutical		
Valoctocogene roxaparvovec (Roctavian, previously known as valrox and BMN-270) AAV5-hFVIII-SQ	Study BMN270-201: A Phase 1/2, Dose-Escalation, Safety, Tolerability, and Efficacy Study of Valoctocogene Roxaparvovec, an Adenovirus-Associated Virus Vector-Mediated Gene Transfer of Human Factor VIII in Patients With Severe Hemophilia A (NCT02576795) EudraCT Number: 2014–003880-38	Phase 1/2 study – active, not recruiting 15 subjects Results published [98,126,159,160]
	Study BMN 270–203: A Phase 1/2 Safety, Tolerability, and Efficacy Study of Valoctocogene Roxaparvovec, an Adeno-Associated Virus Vector-Mediated Gene Transfer of Human Factor VIII in Hemophilia A Patients with Residual FVIII Levels ≤ 1 IU/dL and preexisting Antibodies Against AAV5 (NCT03520712)	Phase 1/2 – enrolling 10 subjects planned
	Study BMN 270–301: A Phase 3 Open-Label, Single-arm Study to Evaluate the Efficacy and Safety of BMN 270, an Adeno-Associated Virus Vector-Mediated Gene Transfer of Human Factor VIII in Hemophilia A Patients with Residual FVIII Levels ≤1 IU/dL (NCT03370913)	Phase 3 – active, not recruiting 134 subjects planned [160]
	Study BMN270-302: Phase 3 Study To Evaluate Efficacy/Safety of Valoctocogene Roxaparvovec an AAV Vector-Mediated Gene Transfer of hFVIII at a Dose of $4x10^{13}$vg/kg in Hemophilia A Patients with Residual FVIII Levels ≤1 IU/dL Receiving Prophylactic FVIII Infusions (GENEr8-2) (NCT03392974).	Phase 3 – active, not recruiting 40 subjects planned
	Study BMN 270–303: A Phase 3b, Single Arm, Open-label Study to Evaluate the Efficacy and Safety of BMN 270, an Adeno-Associated Virus Vector-Mediated Gene Transfer of Human Factor VIII, with Prophylactic Corticosteroids in Hemophilia A Patients (GENEr8-3) (NCT04323098) EudraCT Number: 2018–004616-21	Phase 3b – not yet recruiting 20 subjects planned
University College London (UCL)		
AAV2/8-HLP-FVIII-V3	Study UCL 13/0076: GO-8: Gene Therapy for Hemophilia A Using a Novel Serotype 8 Capsid Pseudotyped Adeno-associated Viral Vector Encoding Factor VIII-V3 (NCT03001830)	Phase 1 – recruiting 18 subjects planned
Spark Therapeutics		
SPK-8011 rAAV-LK03	Study SPK-8011-101: Gene Transfer, Dose-Finding Safety, Tolerability, and Efficacy Study of SPK-8011 (a Recombinant Adeno-Associated Viral Vector with Human Factor VIII Gene) in Individuals with Hemophilia A (NCT03003533)	Phase 1 – recruiting 30 subjects planned
	Study SPK-8011-LTFU: A Multi-Center Evaluation of the Long-Term Safety and Efficacy of SPK-8011 (Adeno-Associated Viral Vector with B-Domain Deleted Human Factor VIII Gene) in Males With Hemophilia A (NCT03432520)	Observational study, long term follow-up – enrolling by invitation 100 subjects planned
SPK-8016	Study SPK-8016-101: Dose-finding Study of SPK-8016 Gene Therapy in Patients with Hemophilia A to Support Evaluation in Individuals with FVIII Inhibitors (NCT03734588)	Phase 1/2 – active, not recruiting 30 subjects planned
Pfizer/Sangamo Therapeutics		
SB-525 PF-07055480 Giroctocogene fitelparvovec	Study SB-525-1603: A Phase 1/2, open-label, adaptive, dose-ranging study to assess the safety and tolerability of SB-525 (PF-07055480) (recombinant AAV2/6 human factor 8 gene therapy) in adult subjects with severe hemophilia A (Alta Study) (NCT03061201)	Phase 1/2 – recruiting 13 subjects planned
	Study C0371004: An Open-Label, Non-investigational Product, Lead-in Study to Evaluate at Least 6 Months of Prospective Efficacy and Safety Data of Factor IX or Factor VIII Prophylaxis Replacement Therapy in the Usual Care Setting of Moderately Severe to Severe Adult Hemophilia B Subjects (FIX:C ≤ 2%) who are Negative for Nab To AAV Vector-Spark100 and Moderately Severe to Severe Hemophilia A Adult Subjects (FVIII:C ≤ 1%) who are Negative for Nab to AAV Vector Sb-525 Capsid (AAV6), Prior to the Respective Therapeutic Ph 3 Gene Therapy Studies (NAB Protocol) (NCT03587116) EudraCT Number: 2017–001271-23	Phase 3 – recruiting 250 subjects The data obtained from this 6-month lead-in study will serve as the control group for the subsequent Phase 3 study.
	Study C3731003: Phase 3, Open-Label, Single-Arm Study to Evaluate the Efficacy and Safety of PF-07055480 (Recombinant AAV2/6 Human Factor VIII Gene Therapy) in Adult Male Participants With Moderately Severe to Severe Hemophilia A (FVIII:C ≤ 1%) (AFFINE) (NCT04370054) EurdaCT Number: 2019–004451-37	Phase 3 pivotal – recruiting 63 subjects planned
Bayer/Ultragenix Pharmaceutics		
BAY 2599023 (DTX 201)	Study 19,429: A Phase 1/2 Open-label Safety and Dose-finding Study of BAY2599023 (DTX201), an Adeno-associated Virus (AAV) hu37-mediated Gene Transfer of B-domain Deleted Human Factor VIII, in Adults With Severe Hemophilia A (NCT03588299) EudraCT Number: 2017–000806-39	Phase 1/2 – recruiting 30 subjects planned Results presented at Scientific Meeting: [165]

(*Continued*)

Table 2. (Continued).

Product	Study number/title (**ClinicalTrial.gov identifier number**)	Status
Takeda/Shire (Baxalta)		
TAK-754 (previously SHP654 and BAX 888)	Study 201,501: A Global, Open-Label, Multicenter, Phase 1/2 Study of the Safety and Dose Escalation of BAX 888, an Adeno-Associated Virus Serotype 8 (AAV8) Vector Expressing B-Domain Deleted Factor VIII in Severe Hemophilia A Subjects Administered a Single Intravenous Infusion (NCT03370172) EudraCT Number: 2015–005576-22	Phase 1/2 – active, not recruiting 12 subjects
Shenzhen Geno-Immune Medical Institute		
YUVA-GT-F801	Study GIMI-IRB-17007: Lentiviral FVIII Gene Therapy for Hemophilia A (NCT03217032)	Phase 1 – not yet recruiting 10 subjects planned
Expression Therapeutics		
CD34+ hematopoietic stem cells transduced with CD68-ET3 lentiviral vector	Study ET3-201: Phase 1 Study of Hematopoietic Stem Cell Transplantation (HSCT) Gene Therapy Incorporating a Lentiviral Vector (LV) Encoding a High Expressing Factor VIII Transgene for Treatment of Severe Hemophilia A (NCT04418414)	Phase 1 – not yet recruiting 7 subjects planned

Source: www.clinicaltrials.gov (date accessed: 20 April 2021)

(referred to as HSQ, and most commonly used in current clinical studies for severe hemophilia A).

A Phase 1/2 clinical dose-escalation study (Study BMN270-201, NCT02576795) to evaluate the effects of a single administration of one of several doses with a 3-year follow-up was conducted in 15 men with severe hemophilia A [98,126]. Overall, the treatment substantially reduced the ABR, allowing subjects that received 4×10^{13} or 6×10^{13} vector genomes (vg)/kg body weight of AAV5–hFVIII-SQ to discontinue prophylactic FVIII use. During the 3-year follow-up after the single administration, none of the participants had developed inhibitors, thromboses, or showed persistent changes in liver-function tests, and none died [126]. An additional year of data (cutoff 8 April 2020) on this cohort was presented at the World Federation of Hemophilia (WFH) for the 6×10^{13} vg/kg cohort as well as 3 years of data for the 4×10^{13} vg/kg cohort.

The 6 subjects in the 6×10^{13} vg/kg AAV5–hFVIII-SQ cohort who had received FVIII prophylaxis prior to the AAV5–hFVIII-SQ treatment exhibited remarkable and long-lasting reductions in bleeding episodes that required an FVIII infusion. The cumulative mean ABR during the 4 years following AAV5–hFVIII-SQ treatment was 0.8, indicating a 95% decrease from that the year before the trial (mean baseline ABR = 16.3, median = 16.5). At 4 years, the mean ABR for all 6 subjects was 1.3 (median = 0). In this group, FVIII usage could be reduced overall by 96% during the 4-year study period from a baseline mean of 135.6 infusions/year to a mean of 5.4 infusion/year. Among the 7 subjects in this cohort, 6 (86%) experienced no bleeding episodes in year 4. None of the 7 subjects currently requires FVIII prophylaxis therapy.

The 6 subjects in the 4×10^{13} vg/kg AAV5–hFVIII-SQ cohort also exhibited long-lasting reductions in bleeding events that required FVIII infusions. All 6 subjects were able to discontinue FVIII prophylaxis therapy. The cumulative mean ABR during the 3 years following treatment was 0.9, indicating a 95% decrease compared with that the year before starting the AAV5–hFVIII-SQ treatment (mean ABR = 12.2, median = 8.0), and 5 of the 6 participants experienced no target joint bleeds in year 3 of the follow-up. In year 3, the mean ABR was 0.5 (median = 0), and no bleeding events occurred in 4 of the 6 subjects. Among the 6 subjects, 5 reported no spontaneous bleeding events. In this group, the number of FVIII infusions over the 3 years was reduced by 96%, from 142.8 infusions/year at baseline to 5.7 infusions/year after 3 years.

The mean FVIII activity levels at the end of the evaluation period in both dose cohorts support the decreases in the ABR and number of FVIII infusions. At the end of the study period, all of the subjects continued to produce their own endogenous FVIII. In the 6×10^{13} vg/kg cohort, the mean (median) FVIII activity was 24.2 (16.4) IU/dL (chromogenic substrate assay) and 35.4 (23.4) IU/dL (one-stage assay). In the 4×10^{13} vg/kg cohort, the mean (median) FVIII activity was 9.9 (7.9) IU/dL (chromogenic substrate assay) and 14.9 (12.3) IU/dL (one-stage assay).

Treatment with AAV5–hFVIII-SQ therapy is now being tested in additional Phase 1/2 and Phase 3 clinical studies (Table 2). A global Phase 3 study of AAV5–hFVIII-SQ at the 6×10^{13} vg/kg dose (GENEr8-1, Study BMN 270–301, NCT03370913, n = 134 participants) to compare the efficacy of AAV5–hFVIII-SQ to the current standard of care, FVIII prophylactic therapy, is in progress. Enrollment is completed and the data from at least 1 year of follow-up indicate an 84% reduction in mean ABR and a 99% reduction in the mean annualized FVIII infusion rate. At 1 year after treatment, the mean FVIII expression level was 42.9 IU/dL.

BioMarin is also conducting a Phase 1/2 study in subjects (n = 10) with preexisting AAV5 antibodies using the 6×10^{13} vg/kg dose of AAV5–hFVIII-SQ (Study BMN 270–203, NCT03520712). In addition, BioMarin is conducting 2 other studies: an evaluation of the seroprevalence of AAV in people with severe hemophilia A, and a non-interventional study aiming to establish baseline characteristics of people with hemophilia A. Because of differences between the Phase 1/2 and Phase 3 studies, the durability of the AAV5–hFVIII-SQ treatment is not clear [161].

3.2.2. University College London (UCL)

The University College London study is an ongoing Phase 1/2 open label clinical study evaluating treatment with an AAV vector expressing a 17-amino acid peptide containing 6 N-linked glycosylation motifs from the human FVIII B-domain

with a liver-specific transporter (AAV8-HLP-hFVIII-V3; GO-8, Study UCL 13/0076, NCT03001830). The study utilized relatively low doses of AAV8-HLP-FVIII-V3 compared with other related FVIII gene therapies. Preliminary results published in 2018 [162] revealed that all 3 participants had FVIII activity levels under 5% and 1 participant had normal procoagulant activity (FVIII:C) levels. Spontaneous hemorrhage events were reduced or prevented during the preliminary observation period. Adverse events of Grade 3 or higher were not reported during the first 47 weeks after treatment [162].

3.2.3. Spark Therapeutics

Spark Therapeutics is also evaluating the efficacy of reduced-size FVIII cassettes on FVIII production in patients with hemophilia A. Two different constructs, SPK-8011 and SPK-8016, are being evaluated. SPK-8011 (rAAV-LK03 vector) is a recombinant AAV vector containing a codon-optimized human FVIII gene controlled by a liver-specific promoter. SPK-8016 is an internally developed gene therapy. Three clinical studies, 2 evaluating SPK-8011 and 1 evaluating SPK-8016, are in progress [163].

In the Phase 1/2 open-label, non-randomized study with SPK-8011 in subjects with hemophilia A (the data cutoff date was May 3, 2021, NCT03003533), a single dose of SPK-8011 (dose cohorts ranged from 5×10^{11} to 2×10^{12} vg/kg) was administered to a total of 18 subjects in 4 cohorts: $5X10^{11}$ (N= 2), $1X10^{12}$ (n= 3), $1.5X10^{12}$ (n= 4) and $2x10^{12}$ (n= 9) [164]. Sixteen participants demonstrated sustainable FVIII expression, stopped baseline prophylaxis and demonstrated a 91.5% reduction in ABR and 96.4% reduction in the annualized number of FVIII infusions. Two participants lost FVIII expression, presumably the result of a capsid-based immune response.

The second clinical trial of SPK-8011 will monitor the safety and efficacy (for up to 5 years) of a single dose of SPK-8011 in approximately 100 men with hemophilia A that participated in a previous Spark-sponsored a SPK-8011 study (Study SPK-8011-LTFU, NCT03432520).

Spark Therapeutics is also conducting a Phase 1/2, open-label, non-randomized, dose-finding study for SPK-8016 (Study SPK-8016-101, NCT03734588) in adult men with clinically severe hemophilia A who have not developed FVIII inhibitors. The safety, efficacy, and tolerability of SPK-8016 in adult men with clinically severe hemophilia A who have not developed FVIII inhibitors will be evaluated in Part 1, and the data from Part 1 will be used to design and select the dose for Part 2 in adult men who have developed FVIII inhibitors. Preliminary data from 4 participants show that FVIII was consistent and durable over 52 weeks (ranging from 5.9%–21.8%) in the 5×10^{11} vg/kg cohort, with a 98% reduction in annualized infusion rate and 85% reduction in ABR.

3.2.4. Pfizer/Sangamo Therapeutics

Pfizer is advancing an investigational new drug, giroctocogene fitelparvovec (SB-525 or PF-07055480; originally developed by Sangamo Therapeutics but transferred to Pfizer in 2019) into Phase 3 clinical studies. Giroctocogene fitelparvovec is a recombinant AAV vector that encodes the human FVIII gene from which the B domain has been deleted.

Giroctocogene fitelparvovec is being investigated in a Phase 1/2 open-label study (Alta study, Study SB-525-1603, NCT03061201) with 11 male subjects treated across 4 ascending dose cohorts: 9×10^{11} vg/kg (n = 2), 2×10^{12} vg/kg (n = 2), 1×10^{13} vg/kg (n = 2), and 3×10^{13} vg/kg (n = 5). Updated results were presented at the World Federation of Hemophilia 2020 World Congress, held in June 2020, as follows.

The 5 participants in the 3×10^{13} vg/kg giroctocogene fitelparvovec cohort exhibited a sustained increase in the FVIII activity levels (median 64.2%), no bleeding events, and did not require FVIII infusions. Giroctocogene fitelparvovec was generally well tolerated with only 1 participant in the highest (3×10^{13} vg/kg) dose cohort experiencing treatment-related serious adverse events of hypotension (Grade 3) and fever (Grade 2) occurring within 6 h of infusion (fully resolving within 24 h). Of the 5 participants in the 3×10^{13} vg/kg dose cohort, 4 were treated with oral corticosteroids due to elevated liver enzymes (ALT), which fully resolved with treatment.

Pfizer is also currently enrolling subjects in a Phase 3 lead-in study (NAB Protocol, Study C0371004, NCT03587116) in which no investigational product is being administered (only standard of care replacement therapy), the data from which is expected to provide a baseline for subjects who are subsequently enrolled into a pivotal Phase 3 study (AFFINE Study, Study C3731003, NCT04370054). The Phase 3 study will primarily evaluate ABR with secondary endpoints including steady state FVIII activity levels, annualized FVIII infusion rates, annualized FVIII consumption, cause and location of ABR, and joint health changes, over a 12-month period. This is an ongoing study in which the first participant was dosed in October 2020.

3.2.5. Bayer/Ultragenix Pharmaceutical

BAY 2599023 is being developed by Bayer in collaboration with Ultragenyx Pharmaceutical. BAY 2599023 is an AAV vector encoding FVIII with the B-domain deleted that is controlled by a liver-specific promoter and enhancer optimized for transgenic expression. The ongoing, dose-establishing Phase 1/2 study (Study 19,429, NCT03588299) is evaluating the safety, tolerability, and early effectiveness of 3 ascending doses of BAY 2599023 in men with severe hemophilia A that were previously treated with FVIII products. Preliminary data presented at the American Society of Hematology Annual Meeting (December 2020) indicated that BAY 2599023 delivered early measurable FVIII expression levels that were sustained (>18 months) with evidence of hemostatic efficacy across 3 dose cohorts (0.5×10^{13}, 1×10^{13}, and 2×10^{13} gene copies/kg [n = 2 each]) [165]. Several patients (cohorts 2 and 3), who were all on FVIII prophylaxis prior to gene therapy, have been off prophylaxis since approximately 6 weeks after gene transfer. Those participants that achieved FVIII levels ≥15 IU/dL reported no spontaneous bleeding events. Both subjects in cohort 3 had elevated ALT levels (>1.5 times the upper limit of normal) and were treated with corticosteroids. The study is currently enrolling subjects (up to 30 eligible adult subjects).

3.2.6. Takeda/Shire

Takeda's gene therapy pipeline for hemophilia includes TAK-754 (previously known as SHP654 and BAX 888), which is an AAV serotype 8 vector that expresses FVIII with the B domain

deleted, for hemophilia A. They are conducting a Phase 1 clinical study (Study 201,501, NCT03370172) that is active but not recruiting.

3.2.7. Shenzhen Geno-immune Medical Institute

In 2017, Shenzhen Geno-immune Medical Institute registered a clinical study of lentiviral FVIII gene-modified autologous hematopoietic stem cells and mesenchymal stem cells (Study GIMI-IRB-17007, NCT03217032), but as of June 2021, the study remains in non-recruiting status.

3.2.8. Expression Therapeutics

Expression Therapeutics registered a clinical study of hematopoietic stem cell transplantation gene therapy incorporating a lentiviral vector encoding the highly expressing FVIII transgene ET3 (Study ET3-201) for the treatment of severe hemophilia A (NCT04418414), but as of June 2021, the study remains in non-recruiting status.

4. Second-generation gene therapy, ASC618

4.1. ASC618 construct

Applied StemCell (ASC) Therapeutics, Inc. has developed a construct called ASC618 (AAV2/8 HCB-ET3-LCO BDD FVIII viral vector), which is a hybrid AAV2/8 vector encoding BDD, codon-optimized hFVIII (BDD hFVIII) with a synthetic liver-directed promoter (Figure 3). Compared with other clinically tested gene therapy constructs, ASC618 has the shortest vector genome. The construct includes a liver-specific, codon-optimized (LCO) bioengineered BDD hFVIII (ET3) controlled by a synthetic hepatic combinatorial bundle (HCB) promoter.

ASC618 is designed to express the hFVIII protein to treat patients with severe and moderately severe hemophilia A. ASC618 is supplied frozen as a viral vector in individual vials and is administered by a single intravenous infusion.

Both rational and empirical design strategies were applied to produce ASC618, a minimally-sized, highly potent AAV-FVIII vector incorporating 2 unique elements: 1) a minimal liver-directed HCB promoter (146 bp) to minimize the packaging size and allow for higher protein expression levels; and 2) a novel bioengineered FVIII molecule, ET3, with 10- to 100-fold increased biosynthesis, expression, and secretion efficiency compared with standard hFVIII transgenes (known as HSQ; the BDD hfVIII protein that contains the 14-amino acid human-derived SQ linker sequence instead of the B-domain) used currently in most hemophilia A gene therapies. Expression Therapeutics/Emory University characterized the HCB-ET3-LCO construct in a murine model of hemophilia A and licensed it to ASC Therapeutics for further therapeutic development.

The ASC618 construct uses a chimeric human/porcine FVIII molecule, ET3, to enhance vector potency. ET3 is a bioengineered human BDD FVIII protein with porcine A1 and A3 domain elements (91% human, 9% porcine) [86,145,149,157,166,167]. The novel bioengineered FVIII molecule ET3 (previously referred to as HP47) was developed based on BDD recombinant porcine FVIII [149,150]. Recombinant porcine FVIII (rpFVIII, Obizur®) was originally developed at Emory University and approved by the FDA for the treatment of acute bleeds in patients with acquired hemophilia A [168,169]. Extensive work was performed to optimize the pharmaceutical properties of FVIII protein by investigating FVIII sequences across different ancestral species (ancestral sequence reconstruction approach). FVIII protein variants were engineered with superior properties as compared with current hFVIII biologics, including improved activity, stability, biosynthesis potential, and reduced inhibition by clinical anti-drug antibodies [170].

Because the rpFVIII and ET3 molecules interact less with the ER resident chaperones and are thus less likely to induce the UPR, they are secreted much more efficiently than other FVIII constructs [145,149,150,171]. In ET3, more highly expressing rpFVIII sequences are substituted into the A1 and *ap*-A3 domains of the recombinant human FVIII. This small sequence modification (~9%) accounts for the remarkable 10- to 100-fold improved biosynthesis [149].

4.2. Liver-specific codon optimization

Compared with standard genome-level codon optimization strategies, tissue-directed codon-optimization strategies enhance the expression of FVIII transgenes in specific cell types (e.g. hepatocytes). All current rAAV-FVIII product candidates that are undergoing clinical trials utilize codon-optimized transgenes. In the traditional codon-optimization strategies, the codon usage bias of the whole organism is derived from whole genome cDNA, which was assumed to represent the concentrations of the transfer ribonucleic acid within each individual cell. In fact, transfer RNA concentrations of individual cells vary considerably among various tissue and cell types [172].

Using this novel codon optimization strategy, Doering and colleagues [166] examined tissue/cell type-specific codon usage bias tables for codon optimization in liver-directed

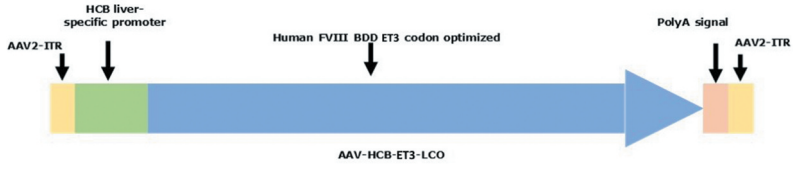

Figure 3. **ASC618 Schematic Diagram Expression Cassette.** The ASC618 construct consists of an AAV8 vector encoding a codon-optimized hFVIII gene that has been minimized by deleting the B-domain (BDD). The construct is designed to be specifically expressed in liver cells.

AAV gene therapy to further improve the expression of a BDD hFVIII (HSQ) and ET3. When transfected into HepG2 cells, ET3-LCO exhibits significantly higher expression than either the myeloid codon optimization or native non-codon-optimized cognates [166]. The effect of liver codon optimization was also confirmed in vivo in a hemophilia A mouse model when ET3-LCO provided a 3- to 4-fold increase in expression over both ET3-myeloid codon optimization and ET3-non-codon–optimized [166].

4.3. HCB promoter

The optimal capacity of the rAAV vector genome is approximately 4.7 to 4.9 kb [93,157,173]. The rAAV-FVIII vectors generally exceed this ideal vector genome length because of the large size of the transgene and the need for non-coding viral and gene expression regulatory control elements, which results in inadequate transgene packaging and delivery.

At a minimum, the rAAV-FVIII genome must include a promoter, the FVIII transgene, a polyA signal, and rAAV inverted terminal repeats flanking both sides of the cassette. The inverted terminal repeats and the FVIII transgene alone require 4664 bp of the available 4900 bp; thus, the promoter, polyA signal, and any other required sequences must fit into the remaining 246 bp. Doering and colleagues [166] used both random combinatorial and in silico rational design approaches to address this limitation by generating synthetic promoters that are more compact than existing promoter and yet are still able to drive strong expression in hepatocytes while retaining equivalent or superior transcriptional output. After 3 sequential rounds of design/optimization, they identified a 146-bp synthetic promoter, named HCB, that drives 20-fold greater FVIII production than the established gold-standard HLP promoter while being more than 100 bp shorter. HCB testing comprised transient transfection of the human liver hepatocellular carcinoma cell line HepG2, and hydrodynamic injection of naked plasmid DNA encoding the respective AAV genome into hemophilia A model mice [166].

4.4. Comparison between ASC618 and other investigational products

The enhanced biosynthesis of the ET3 transgene confers significantly better therapeutic potential than standard hemophilia A gene therapies, based on pre-clinical studies (Figure 4).

Comparison of the effect of the ASC618 transgene vs the standard transgene on FVIII activity. Three transgenes, AAV2/8 HCB-HSQ-LCO, AAV2/8-HCB-ET3-LCO, and AAV2/8-HLP-V3co, were administered intravenously at a dose of 1×10^{11} vg/kg to a mouse model of hemophilia A (n = 4/group). Plasma FVIII activities over the course of 16 hours were measured. The ASC618 transgene produced significantly greater FVIII activity than the standard and control transgenes (Fig. 6 C from Brown et al. [166] replicated with permission from the publisher).

ET3 and HSQ were compared in nonclinical safety and efficacy experiments in a C57Bl/6 murine model, a cynomolgus monkey model, and a humanized liver mouse model (FRG-KO). In all 3 models, the vector containing the ET3 transgene produced higher FVIII levels compared to the vector containing the HSQ transgene [174].

In the C57Bl/6 murine model, AAV2/8 HCB-ET3-LCO at doses of 5×10^{10}, 5×10^{11}, and 5×10^{12} vg/kg led to stable human FVIII expression with mean ET3 FVIII levels as high as 50% (0.5 IU/mL), 300% (3 IU/mL), and 350% (3.5 IU/mL) of normal, respectively. On the other hand, treatment with AAV2/8 HCB-HSQ-LCO at doses of 5×10^{11} and 5×10^{12} vg/kg produced HSQ FVIII expression levels that were 7-fold and 3-fold lower, respectively, and no HSQ expression was detected at a dose of 5×10^{10} vg/kg. In the cynomolgus monkey experiments, the trend was similar; AAV2/8 HCB-ET3-LCO at a dose of 5×10^{11} vg/kg induced expression levels almost 30% (0.3 IU/mL) that of normal. In the humanized liver FRG-KO model, ET3 treatment at a dose of 3×10^{12} vg/kg dose induced mean expression levels of human FVIII levels as high as 480% (4.8 IU/mL) that of normal, compared with only approximately 30% after HSQ treatment. Moreover, in the FRG-KO model of human hepatocytes, ASC618 administration resulted in high ET3 mRNA expression, as assessed by RNAScope analysis. In all 3 models, safety studies, including clinical observations; measurements of food consumption,

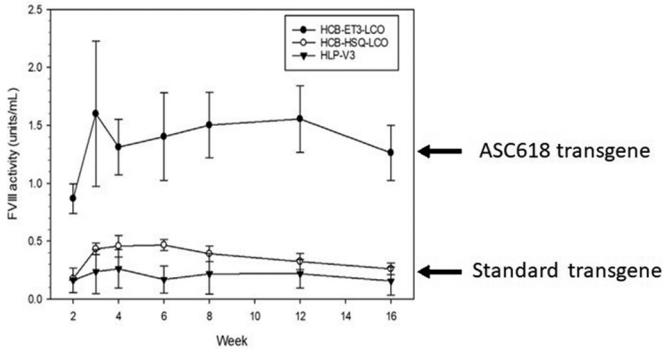

Figure 4. AAV Vector Delivery of Tissue-Optimized Transgene Cassettes.

body weight, and temperature; and evaluation of liver enzymes and gross pathology, demonstrated no toxicity. Thus, ASC618 was well-tolerated in animal models and demonstrated the potential to provide a therapeutic benefit to patients at reduced vector doses [174].

5. Expert opinion

Hemophilia is well-studied as a gene therapy target. Both pre-clinical and clinical data point toward the potential of gene therapy to improve the patients' quality of life by inducing sufficient synthesis and secretion of FVIII to normalize clotting factor activity. Certain limitations, however, prevent currently available gene therapies from becoming a definitive cure for all patients. A number of factors influence treatment durability and long-term safety, especially the development of anti-AAV neutralizing antibodies, transgene product inhibitors, hepatoxicity, cellular stress, and the potential for tumorigenesis.

Aiming to increase treatment durability, intensive non-clinical and clinical research is focusing on the causes and amelioration of cellular stress in hepatocytes driven by post-translational folding of the FVIII protein. Comprehensive assessments of demographic, genetic, and other individual factors are also needed to understand the significant variability in FVIII activity observed among treated patients.

Next-generation gene therapies should improve the synthesis and secretion of FVIII, while limiting the development of anti-AAV neutralizing antibodies and development of cellular stress. This goal can be achieved by transgene engineering strategies that maximize transgene expression while minimizing the post-translational cellular stress that can potentially lead to apoptosis of the transfected hepatocytes. Pre-clinical studies of a chimeric human-porcine construct in wild-type and humanized liver mice and non-human primates demonstrated both a 10- to 100-fold increase in FVIII synthesis and reduced cellular stress. Clinical studies of a human-porcine chimeric FVIII transgene (ASC618, NCT04676048) will be initiated shortly and may confirm the findings from pre-clinical studies showing that, compared with a fully human factor VIII transgene, the human-porcine chimeric transgene allows for lower AAV doses while still generating sufficient serum levels of FVIII and a more durable treatment effect.

Advancing the field of gene therapy will require a better understanding of the target cells e.g. the physiology of the hepatocyte as a biofactory for FVIII. Elucidation of the factors influencing the transcription, translation, post-translation, and secretion of a protein will have critical implications for improving the efficacy, safety, and, especially, the durability of gene therapies. Future gene replacement therapies must meet the challenges of prolonging the durability of transgene expression and enhancing the efficacy of the therapy in children. Current gene replacement therapies are limited because the transgene does not replicate within the cell and thus, transgene expression is diluted and lost over time; this is particularly relevant to children. ASC Therapeutics is currently pursuing a gene-editing program using a CRISPR/Cas9-based in vivo genome-editing. This method incorporates non-homologous end-joining that enables permanent chromosomal integration of a modified human B-domain–deleted FVIII at the albumin locus in liver cells to prevent the loss of AAV vector due to hepatocyte proliferation. Such an approach may revolutionize the treatment of hemophilia in young patients who are currently not eligible for standard gene therapy.

Advances in the mechanistic understanding of transgene insertion into hepatocytes will greatly improve our understanding of liver-targeted gene therapies for other indications and liver diseases, as well, such as nonalcoholic fatty liver disease, alcoholic liver disease, and hepatitis. Producing a safe, durable, and stable therapy to replace or supplement missing or defective proteins for a wide range of conditions while also decreasing the societal and patient burden is the ultimate goal of gene therapy, and recent progress in this field is providing promising advances for the near future.

Abbreviations

AAV, Adeno-associated virus; ABR, Annualized bleeding rate; ALT, Alanine aminotransferase; bp, Base-pairs; Cas9, CRISPR-associated protein 9; CRISPR, Clustered regularly interspaced short palindromic repeat; ER, Endoplasmic reticulum; FVIII, Factor VIII; FIX, Factor IX; HCB, Hepatic combinatorial bundle; ITI, Immune tolerance induction; kb, Kilobases; rAAV, Recombinant AAV; UCL, University College London; UPR, Unfolded protein response; US, United States; vg, vector genome; vWF, von Willebrand factor; wt, Wild-type

Acknowledgments

We thank Steve Zhang, PhD, for his comments on the gene-editing section. Pazit Flint-Richter, PhD and Osnat Carmi-Nir, PhD from TLR Tech LLC (San Diego, CA), and Karin Mesches, PhD and Michael Mesches, PhD from SciTechEdit International LLC (Highlands Ranch, CO) provided support in preparing the manuscript.

Declaration of Interests

SW Pipe has served as a consultant to ASC Therapeutics. G Gonen-Yaacovi and OG Segurado are employees of ASC Therapeutics, a biopharmaceutical company headquartered in Milpitas, CA, USA. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Reviewer disclosures

One of the peer reviewers on this manuscript chairs a Data Monitoring Committee for hemophilia A gene therapy clinical trials. Peer reviewers on this manuscript have no other relevant financial relationships or otherwise to disclose.

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