Molecular Therapy
Clinical Perspective

Clinical perspective: Advancing hemophilia treatment through gene therapy approaches

Courtney D. Thornburg,[1] Steve W. Pipe,[2] Alessio Cantore,[3,4] Carmen Unzu,[5] Micheala Jones,[6] and Wolfgang A. Miesbach[7]

[1]National Institutes of Health, National Heart, Lung, and Blood Institute, Division of Blood Diseases and Resources, Bethesda, MD, USA; [2]Pediatric Hematology-Oncology, University of Michigan, Ann Arbor, MI, USA; [3]San Raffaele Telethon Institute for Gene Therapy, IRCCS San Raffaele Scientific Institute, Milan, Italy; [4]Vita-Salute San Raffaele University, Milan, Italy; [5]DNA and RNA Medicine Division, CIMA Universidad de Navarra, Pamplona, Spain; [6]Regeneron Pharmaceuticals, Inc., Tarrytown, NY, USA; [7]Department of Haemostaseology University Hospital Frankfurt, Frankfurt, Germany

Hemophilia, a congenital bleeding disorder, can cause arthropathy, impaired mobility, pain, and life-threatening hemorrhage events, significantly impacting quality of life for patients and caregivers. Current therapies, although effective, necessitate costly lifelong treatment, often in specialized settings. However, as a monogenic disorder caused by loss-of-function genetic variants, hemophilia is amenable to gene therapy. In this article, three primary gene therapy approaches at the forefront of clinical development are reviewed. Adeno-associated virus-based gene therapy, having secured approval in the EU, UK, and US after promising phase 3 trial results, demonstrates clear superiority over standard-of-care treatment. Lentivirus-based approaches capable of transducing dividing and nondividing cells may improve the durability of treatment and have low susceptibility to pre-existing neutralizing antibodies to viral vectors. Finally, gene editing techniques such as zinc finger nucleases and CRISPR aim to correct genetic defects directly, holding promise as novel, effective, and highly durable therapeutic strategies in adults and children with hemophilia. This review provides a comprehensive summary of the current status of these gene therapy approaches, highlighting advantages, limitations, and potential future developments.

INTRODUCTION

Hemophilia is a congenital bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII; hemophilia A) or factor IX (FIX; hemophilia B),[1,2] and as a monogenic disorder caused by loss-of-function mutations,[1] hemophilia is amenable to gene therapy. Hemophilia has an estimated prevalence of approximately 1 in 10,000 live births,[1] with hemophilia A being more prevalent (estimated prevalence at birth is 24.6 cases per 100,000 male individuals[3,4]) than hemophilia B (5.0 cases at birth per 100,000 male individuals[3]). The X-linked recessive pattern of hemophilia A and B means most persons with hemophilia are male, although small numbers of female individuals are also affected due to inactivation or absence of an X chromosome or both parents carrying hemophilia-associated genetic variants.[1]

Bleeding events have considerable short- and long-term effects, including complications such as arthropathy and associated chronic pain (as a consequence of intra-articular bleeding).[1,2,5] The ever-present risk of bleeding events can result in lifestyle considerations, such as whether to participate in sports involving physical contact or high-velocity impact.[1,6] Moreover, intracranial, thoracic, pharyngeal, and post-surgical bleeds can be life-threatening.[1,2] Beyond bleeding and the associated complications, severe hemophilia can impact many aspects of daily living and family life.[4] Individuals with severe hemophilia and their caregivers may face several psychosocial and economic burdens, including worsened quality of life and negative impacts on mental health and relationships.[6,7] To prevent and manage bleeding events and optimize health outcomes, individuals with severe hemophilia (factor activity levels <1 IU/dL) require lifelong management and individuals often need treatment in specialized healthcare settings.[4]

With an improved understanding of coagulation biochemistry, recent decades have seen advances in the safe and effective treatment of individuals with hemophilia: from several generations of replacement clotting factor concentrates (CFCs) for treatment and prophylaxis, to nonfactor replacement and hemostatic rebalancing therapies for prophylaxis.[4] While traditional treatments involving CFC replacement for prevention and management of bleeding are effective, these therapies are limited by the need for frequent infusions that sometimes need to be administered in a specialized healthcare setting.[8,9] Another major challenge of CFC replacement therapy is the development of neutralizing antibodies (nAbs) against FVIII or FIX (inhibitors). Inhibitors develop in up to one-third of individuals

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Correspondence: Wolfgang A. Miesbach, Department of Haemostaseology University Hospital Frankfurt, Frankfurt, Germany.
E-mail: miesbach@em.uni-frankfurt.de

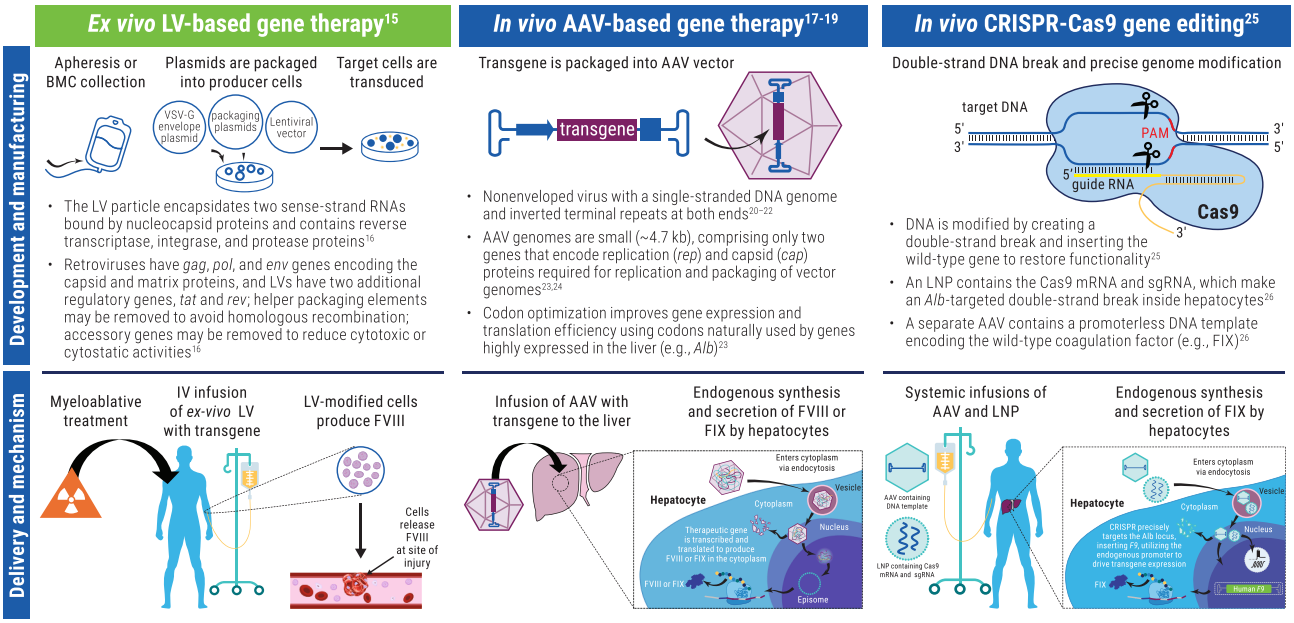

Figure 1. Overview of advanced gene therapies, including general principles of development, manufacturing, and delivery

AAV, adeno-associated virus; Alb, albumin; BMC, bone marrow cell; CRISPR, clustered regularly interspaced short palindromic repeats; FVIII, factor VIII; FIX, factor IX; IV, intravenous; LNP, lipid nanoparticle; LV, lentivirus; mRNA, messenger RNA; nAb, neutralizing antibody; PAM, protospacer-adjacent motif; sgRNA, single guide RNA; VSV-G, vesicular stomatitis virus G protein.

with severe hemophilia and impair the efficacy of treatment, which may necessitate immune tolerance induction and use of alternative treatments to prevent and manage bleeding.[8,9] Subcutaneous bispecific antibody therapy (FVIII mimetic, emicizumab, for hemophilia A)[10] and hemostatic rebalancing therapies (anti-tissue factor pathway inhibitors, concizumab and marstacimab, for hemophilia A or B) achieve sufficient hemostasis to prevent most bleeding even in the presence of FVIII or FIX inhibitors.[10] These treatments may reduce treatment burden through ease and flexibility of administration.[8,9] However, the risk of bleeding is not eliminated[10]; therefore, access to CFC for bleeding management and surgery is still required.[11] Also, the risk of bleeding upon trauma is not eliminated. CFC replacement therapy and nonfactor replacement therapy are associated with high costs and adherence burden, particularly when considering the need for lifelong treatment.[8,9]

By addressing the genetic variants affecting coagulation factor genes, gene therapy offers a promising approach to targeting the underlying cause of hemophilia.[12] CFC replacement therapy has peak and trough pharmacokinetics, which can leave patients at risk of bleeding events as plasma factor levels approach trough levels before the next prophylactic dose.[4] In contrast, the aim of gene therapy is to achieve stable plasma levels of coagulation factors via endogenous synthesis of FVIII (hemophilia A) or FIX (hemophilia B) at levels associated with effective hemostasis.[12,13] A single treatment can be administered with curative intent, which if successful, enables individuals to lead a life free from many of the restrictions associated with hemophilia.[9] This review explores the status of the three primary gene therapy approaches at the forefront of hemophilia research and clinical development: lentivirus-based gene therapy, adeno-associated virus (AAV)-based gene therapy, and approaches based on gene editing techniques, such as clustered regularly interspaced short palindromic repeats (CRISPR [referred to as CRISPR-Cas9, where Cas stands for "CRISPR-associated system"]).[14]

By exploring the different approaches and discussing their relative benefits, limitations, and potential future development, we aim to provide a valuable resource to inform clinicians, researchers, and other healthcare partners in their pursuit of more effective and sustainable therapies for hemophilia. The principles, advantages, and limitations of viral vector-mediated gene therapy and gene editing approaches are summarized in the following sections and in Figure 1 and Table 1.

INTRODUCTION TO GENE THERAPY APPROACHES FOR HEMOPHILIA

Lentiviral-based gene therapy

Lentiviruses belong to a genus of the *Retroviridae* family.[16,28] The viral particle encapsidates two sense-strand RNA molecules bound by nucleocapsid proteins and also contains reverse transcriptase, integrase, and protease proteins.[16] Retroviruses can be classified as simple or complex based on their genome organization; human immunodeficiency virus-1 (HIV-1), a lentivirus, is an example of a complex retrovirus.[16] All retroviruses have *gag*, *pol*, and *env* genes that encode for the capsid and matrix proteins, and lentiviruses have two additional regulatory genes, *tat* and *rev*.[16] Lentivirus-derived vectors or

Table 1. Summary of the benefits and challenges of three gene therapy approaches

Ex vivo LV-based gene therapy		*In vivo* AAV-based gene therapy		*In vivo* CRISPR-Cas9 gene editing	
Benefits	Challenges	Benefits	Challenges	Benefits	Challenges
• Capable of transducing dividing and nondividing cells, and has the potential to treat pediatric populations[28] • Low level of antibodies against vector[29] • Relatively large packaging capacity[30]	• Conditioning regimens are associated with significant toxicity-related risks[31] • Semi-random integrations and off-target effects[32] • Currently pediatric patients are ineligible for treatment/participation in clinical trials[15]	• Easily engineered genome and capsid[33] • Considered to be a nonintegrating vector[34] • Long-term data are being accrued, demonstrating efficacy and tolerability[17–19,33]	• High seroprevalence of nAbs against AAV limit efficacy and re-dosing[35] • Variable response and unknown durability[34] • Potential off-target events (integration)[34] • Currently pediatric patients are ineligible for treatment/participation in clinical trials[17,19,36]	• Capable of transducing dividing and nondividing cells, and has the potential to treat pediatric populations[25] • High degree of precision[14,37]	• Potential off-target effects[38] • Potential integration of AAV ITRs into the double-strand break[39] • Other challenges associated with the delivery system[40] • Currently pediatric patients are ineligible for treatment/participation in clinical trials[25]

AAV, adeno-associated virus; CRISPR, clustered regularly interspaced short palindromic repeats; ITR, inverted terminal repeats; LV, lentiviral vector; nAb, neutralizing antibody.

lentiviral vectors (LVs) are widely used for gene transfer in basic and clinical research, with the most frequently used vector systems based on HIV-1.[16] Given the potential risks of generating a replication-competent lentivirus, several modifications have been made to LVs to enhance safety, including removal of *tat* and *rev* in the packaging construct.[16]

LVs can be susceptible to phagocytosis *in vivo*, but incorporation of high amounts of the phagocytosis inhibitor CD47 into the vector surface appears successful in addressing this potential limitation.[30,41] Intravenous administration of the CD47hi LV in adults or newborns elicited stable FIX and FVIII expression from the liver in small and large animal models.[41–43] However, studies on *in vivo* LV administration to the liver remain limited to the preclinical setting, and no clinical trials have been performed to date. Typically, LVs are integrative vectors that carry an inherent risk of oncogenesis resulting from insertional mutagenesis, for example, hematologic malignancies can arise from LV gene transfer into hematopoietic stem/progenitor cells when strong viral promoters are used.[32,44,45] The LV is integrated into the genome, exerting an effect on the chromatin of target cells,[42] and preclinical studies and clinical experience with *ex vivo* hematopoietic stem cell gene therapy have shown the risk of insertional mutagenesis to be present,[44] but low[46,47] and dependent on vector design.[44,48] Moreover, no insertional mutagenesis has been reported *in vivo* in the liver, even when using tumor-prone or tumor-promoted mouse models.[41,46] Nevertheless, this risk mandates careful risk/benefit assessments on a case-by-case basis.

While systemic LV administration for hemophilia is still in the early stages of development,[15,49–52] many advanced-phase clinical trials and commercial products use LVs to transduce hematopoietic stem cells to treat immunohematologic and lysosomal storage diseases.[47] Cell therapy for hemophilia can be performed by *ex vivo* modification of stem cells to express coagulation factors using an LV before transplantation[29]; *ex vivo* LV-based gene therapy employs an autologous gene modification approach in which stem cells are harvested, cultured *ex vivo*, and modified before being reinfused after receipt of a conditioning regimen that favors engraftment.[29] Hematopoietic stem cells can be harvested from either bone marrow or mobilized peripheral blood.[53]

Preclinical studies have shown the feasibility of using LVs for transgene delivery to hematopoietic stem cells, facilitating the expression of FVIII by platelets or other cells.[54,55] Furthermore, results from a phase 1 trial on novel LV vector delivery of engineered FVIII cassettes for hemophilia A—with improved FVIII expression compared with the more commonly used B-deleted FVIII—demonstrated FVIII expression from monocytes.[52]

A conditioning regimen using chemotherapeutic and radiotherapeutic agents is employed to deplete endogenous hematopoietic stem and progenitor cells (HSPCs) in bone marrow niches, allowing engraftment of donor HSPCs.[31] Conditioning regimens remain associated with significant risks that should be carefully balanced with the

potential benefits in people with hemophilia.[31] Busulfan conditioning is typically used off-label for hemoglobinopathy gene therapies, and as with all alkylating agents, it is associated with the risk of short- and long-term toxicities.[56]

Common short-term toxicities include intestinal mucosal damage, increased risk of infection, and bleeding. Long-term toxicities include anemia, alopecia, pancytopenia, skin changes, impaired spermatogenesis, and increased risk of malignancy.[56,57] Therapeutic drug monitoring using pharmacokinetic models can minimize such toxicities.[31,57]

This approach may leverage previous experience with *ex vivo* gene therapy products; however, the downstream processing of LVs requires concentration and purification and is challenged with achieving a high yield of functional units on a large scale.[16,28]

AAV-based gene therapy

The most common viral vectors for *in vivo* gene therapy are based on AAVs, and all products currently approved for gene therapy in hemophilia use AAV vectors.[9,13] The AAV is a nonenveloped single-stranded DNA virus and a member of the *Dependoparvovirus* genus of the *Parvoviridae* family.[20–22] AAV genomes are small (~4.7 kb), comprising only two genes encoding the replication (*rep*) and capsid (*cap*) proteins.[23,24] Therefore, both the genome and capsid are easy to engineer: tissue-specific promoters can be tailored as required, and transgenic complementary DNA (cDNA) codons can be optimized for efficient expression of FVIII or FIX.[23] There are many naturally occurring AAV serotypes, and the capsid can be configured for specific delivery to the cells of target tissues.[58] To produce a recombinant AAV (rAAV) vector, all viral genes are removed from the genome to accommodate the therapeutic expression cassette, and *rep* and *cap* are provided *in trans*.[22] rAAVs are considered nonpathogenic and have low immunogenicity, but the latter depends on the dose and route of administration.[23] rAAV-mediated gene transfer results in an episomally retained transgene and is predominantly nonintegrating. Therefore, rAAV vectors carry reduced risk of insertional mutagenesis.[59] Taken together with the fact that AAVs are replication defective, these findings support the favorable safety profile of this approach and its fast translation into the clinic.[23,24,59]

rAAV vectors are used for gene delivery in several products approved for treating monogenic diseases, including spinal muscular atrophy, hemophilia, and Duchenne muscular dystrophy.[60] The initial development of AAV-based gene therapy was focused on hemophilia B because the coding region of *F9* is smaller and less complex than that of *F8* and packaging capacity with AAV vectors is limited.[23] Efforts to improve AAV-based gene therapy in hemophilia B included use of the *F9* Padua variant—a naturally occurring hyperactive genetic variant of FIX[61]—which when present, enhances FIX activity up to 8-fold with similar AAV vector doses.[61,62] Liver-directed AAV vectors have been developed to optimize FIX or FVIII expression and reduce AAV immunogenicity; several ongoing gene therapy trials are utilizing liver-specific promoters to drive FIX and FVIII expression.[63,64] To overcome the challenge of gene expression durability in hemophilia A, retargeting vectors to liver sinusoidal endothelial cells may prove beneficial[64] as FVIII is naturally produced in these cells (unlike FIX, which is produced in hepatocytes[65]). Additionally, the use of bioengineered FVIII constructs that are less likely to trigger stress responses in hepatocytes[66] and any associated deleterious impact on FVIII expression may improve gene expression durability.[64]

AAV-based gene therapy has been approved for treatment of hemophilia A and B in the EU, UK, and US.[67–72] Promising results from phase 3 trials have shown superiority of AAV-based gene therapy over prophylactic CFC replacement therapy, with treated individuals demonstrating increased (mean and median) coagulation factor activity levels, reduced annualized bleeding rates (ABRs), and reduced CFC consumption for prophylaxis and treatment.[17–19,73]

Gene editing techniques

Gene editing involves correcting the *F8* or *F9* gene variant of the host DNA or supplementation through gene insertion into the host genome. Host DNA is modified by creating a double-strand break and inserting the corrected sequence, restoring the functionality of the gene. Nevertheless, this field is quickly evolving, and novel technologies for gene editing that do not require DNA double-strand breaks for the edit are under development, including prime and base editing.[26,74,75] Two main classes of engineered endonucleases are available: zinc finger nucleases (ZFNs)[76,77] and CRISPR-Cas9.[14]

Zinc finger nucleases

ZFNs are chimeric nucleases comprising sequence-specific DNA-binding modules linked to a DNA cleavage domain.[76] This design enables the construction of DNA-binding proteins that can modify gene expression; by fusing selected ZFN peptides to activation or repression domains, targeted genes can be selectively switched on or off.[77] To create sequence specificity for targeting a given locus, tandem ZFNs are necessary.[78] However, achieving specificity is complex, expensive, and challenging, resulting in a high frequency of off-target cleavage.

ZFN gene editing methods for hemophilia have been tested in preclinical studies, transducing hepatocytes to produce FIX *in vivo*,[79] and durably restoring therapeutic FVIII and FIX activity levels (>3,000 ng/mL) as measured using a chromogenic assay in murine models of hemophilia A and B *in vivo*.[80] Although these promising results led to clinical exploration, the first-in-human clinical study was terminated as FIX CFC usage could not be reduced in the single participant, suggesting the therapy did not induce a meaningful increase in FIX activity levels (as measured using a one-stage assay [OSA]). FIX activity in the participant increased from 1 IU/dL at baseline to 17 IU/dL at day 60, which was attributed to the FIX infusion on the day before the FIX activity level increase was detected, which the participant continued to receive throughout the study, and not the study treatment.[81]

CRISPR-Cas9

Based on the adaptive immunity of bacteria and archaea, whereby protospacer sequences (target sequence of DNA) of bacteriophages and plasmids are integrated into repeat sequences, the CRISPR-Cas9 system is a recent innovation in gene editing. This technology is considered simpler in terms of reprogramming ease and having a more robust performance than other nuclease tools such as ZFN.[82] CRISPR-Cas9 depends on RNA/DNA recognition for genome engineering, which makes the RNA design simpler compared with ZFN.[83] Therefore, this approach may provide ease of programming and engineering,[84] alongside high accuracy and efficiency.[83–85] The CRISPR-Cas9 platform has recently been further refined to replace double-strand breaks with single-stranded nicks, resulting in the base editing and prime editing platforms.[86,87]

As the Cas9 nuclease has the ability to recognize a specific protospacer-adjacent motif (PAM; a short and conserved sequence on the targeted strand of DNA adjacent to the protospacer), the CRISPR-Cas9 system offers flexible targeted cleavages at numerous genomic loci.[78] Therefore, by changing the PAM, potentially any genome sequence could be targeted and manipulated, making CRISPR-Cas9 ideal for high-throughput applications and multiplexing by enabling multiple simultaneous manipulations.[78] Effective genome correction using CRISPR-Cas9 technology has been demonstrated *in vivo* and *ex vivo* in a preclinical hemophilia study in which genes in *F9*-mutant hemophilia B mouse models were corrected, reversing coagulation deficiency in 62.5% of mice.[88] Other studies have corrected coagulation factor gene variants in adult and newborn mice *in situ* by leveraging the albumin promoter to drive FVIII or FIX production.[89–91]

LVs and AAV vectors can be used to deliver the gene editing machinery and donor DNA.[92] LVs have high transducing efficiency, low immunogenicity, and the ability to be pseudotyped, to redirect cellular tropism. LVs are often used for creating disease models, whereas[92] AAV vectors are the most commonly used vector for gene editing.[92] Owing to the small AAV genome size,[93] it is usually necessary to split the CRISPR-Cas9 expression cassette into a dual vector or combine it with a nonviral delivery system, for example, lipid nanoparticles (LNPs),[37] which have been used for permanent knockout.[94] AAVs offer the advantages of serotype specificity and a generally tolerable safety profile, although they have been reported to be toxic at high doses.[93] An additional potential challenge with using AAV vectors is the unintended integration of AAV genomes into CRISPR-induced double-strand breaks.[39] As a solution, rational design of the AAV/transgene construct, such as removing the promoter and start codon, can help mitigate against such unintended integration.[95] In a preclinical hemophilia B model using a promoterless gene-targeting approach, FIX was produced from on-target integration that achieved phenotypic rescue in neonates.[96]

The use of LNPs to deliver the CRISPR-Cas9 messenger RNA and the albumin guide RNA, together with the AAV encoding the *F9* cDNA, successfully enabled accurate genome integration.[12] This LNP-AAV hybrid technique led to transient Cas9 expression and stable FIX expression driven by the endogenous albumin promoter,[27] with transient Cas9 expression potentially reducing the risk of off-target effects in comparison with continuous expression via viral delivery. LNPs are one of the most popular and sophisticated nonviral delivery systems for CRISPR-Cas9-mediated gene editing, offering ease of formulation, high potency, versatility in design, spontaneous self-assembly, and high biocompatibility.[37] Other key advantages of using LNPs include increasing particle stability and circulation time, allowing CRISPR-Cas9 LNPs to reach target tissues and induce efficient on-target gene editing.[37] Additionally, LNPs can be engineered for increased precision of on-target transgene delivery, for example, by including the N-acetylgalactosamine moiety.[97]

STRENGTHS AND CHALLENGES OF GENE THERAPIES

Viral vector-based gene therapies

Hemophilia A

Based on the availability of data, the following summary of seminal published clinical safety and efficacy data is focused on rAAV-based gene therapy. In a 3-year follow-up of the open-label phase 3 GENEr8-1 clinical trial (NCT03370913) of valoctocogene roxaparvovec (AAV5-hFVIII-SQ) for severe hemophilia A, alanine aminotransferase (ALT) elevation was the most frequently reported treatment-emergent adverse event (TEAE).[98] Related to these ALT elevations, a major concern with rAAV-based gene therapy is the need for prolonged immunosuppression after vector administration. In the phase 1/2 valoctocogene roxaparvovec clinical trial (NCT02576795), all participants who received the 6×10^{13} viral genome (vg)/kg dose (now licensed dose) also received glucocorticoid immunosuppression after trial participants had ALT levels 1.5-fold higher than baseline levels.[99] Similarly, 86% of participants in the phase 3 trial had ALT elevations, necessitating glucocorticoid use in most cases with a median duration of 35 weeks[17]; ALT elevations were detected at approximately 8 weeks after valoctocogene roxaparvovec administration and remained elevated in around one-third of individuals at year 2.[17,73,100] Adverse events (AEs) associated with glucocorticoid use included acne, insomnia, Cushing syndrome, and increased weight; serious AEs (SAEs) associated with glucocorticoid use, including steroid-induced diabetes and hypertension, were reported in 3% of participants.[17,73] Serious infusion-related reactions occurred in three participants in the phase 3 GENEr8-1 trial, in addition to an anaphylactic and a hypersensitivity reaction; no thrombotic events or inhibitors were reported and safety remained unchanged at year 3 compared with previous years.[17,98] Hemostatic efficacy was maintained from year 1 with a mean ABR for treated bleeds of 4.8 (standard deviation [SD] 6.5) at baseline vs. 0.97 (SD 3.48) during year 3. At week 156, mean and median FVIII activity levels were 18.4 IU/dL (SD 30.8) and 8.3 IU/dL, respectively. The decrease in mean FVIII activity levels as measured using the chromogenic assay between years 2 and 3 (from 22.9 to 18.4 IU/dL) was lower than the decrease between years 1 and 2 (from 42.8 to 22.9 IU/dL).[98]

In a phase 1/2 trial for giroctocogene fitelparvovec, the most common TEAE was increased ALT and aspartate aminotransferase (AST) that resolved after corticosteroid administration; two serious TEAEs (hypotension and pyrexia) were reported in a participant within 6 h of infusion, which had resolved 24 h post-infusion. Liver masses, thrombotic events, or confirmed inhibitors were not detected in participants.[101] A reduction in mean circulating FVIII activity levels between week 52 (42.6%; range, 7.8%–122.3%) and week 104 (25.4%; range, 0.9%–71.6%), measured using the chromogenic assay for the highest dose level (3×10^{13} vg/kg; $n = 5$), suggests that durability of response may wane over time.[101] In the phase 3 giroctogene fitelparvovec trial, supranormal elevations of FVIII measured (>150%) in some participants and a leg deep vein thrombosis in a single patient led to the clinical trial being placed on hold.[102]

Hemophilia B

AAV-based gene therapies for hemophilia B appear to have more favorable safety profiles compared with that for hemophilia A in terms of TEAEs, serious TEAEs, treatment-related ALT elevation, and associated glucocorticoid use. In the open-label phase 3 HOPE-B study of etranacogene dezaparvovec (NCT03569891[18]; AAV5-hFIXco-Padua) in individuals with severe or moderately severe hemophilia B, the most common treatment-related AEs at the 24-month post hoc follow-up were ALT elevations (17%), headache (15%), influenza-like illness (13%), and AST elevations (9%).[18] Seven participants (13%) had infusion-related AEs, including infusion-related reactions (three patients) and hypersensitivity (one patient), all of which were mild or moderate, occurred on day 1 of the post-treatment period, and resolved.[19] A significant reduction in mean adjusted ABR was observed at 7–24 months post-treatment vs. the lead-in period for all bleeds with etranacogene dezaparvovec ($p = 0.0002$) and for FIX-treated bleeds ($p = 0.0001$). At 24 months post-treatment, mean FIX activity level measured by OSA was sustained at 36.7% (SD 19.0%).[18]

An SAE of arteriovenous fistula thrombosis occurred in a patient enrolled in the open-label phase 1/2 B-AMAZE study of verbrinacogene setparvovec (NCT03369444 and NCT03641703 [both terminated]; AAV2/S3-FRE1-Ti-FIXco1) for severe or moderately severe hemophilia B[103]; the patient was receiving the highest dose of gene therapy (1.3×10^{12} vg/kg) and had FIX activity levels above the normal range, as measured using OSA.[103] Increases in liver ALT/AST levels were the most common treatment-related AEs in the study. Therapeutic expression of FIX activity was observed in all except one of the individuals at a median follow-up of 27.2 months in the phase 1/2 trial of verbrinacogene setparvovec for individuals with hemophilia B.[103]

Pooled data from the pivotal phase 3 study (NCT03861273) and phase 1/2a study (NCT02484092) showed fidanacogene elaparvovec to be generally well tolerated.[33] A total of 28 participants (62%) received glucocorticoids for increased aminotransferase levels or decreased FIX levels (or both) starting between day 11 and day 123 after gene therapy.[33] No infusion-related SAEs, thrombotic events, development of FIX inhibitors, or malignant conditions were observed. Sustained therapeutic FIX expression of 26.9% was also observed in the phase 1/2a study of fidanacogene elaparvovec, as measured using the OSA.[33] The ABR for all bleeding episodes decreased by 71%, from 4.42 (95% confidence interval [CI] 1.80–7.05) at baseline to 1.28 (95% CI 0.57–1.98) after gene therapy, a treatment difference of −3.15 episodes (95% CI −5.46, −0.83; $p = 0.008$).[33]

LV-based gene therapy with hematopoietic stem cells for the treatment of hemophilia is at an earlier stage of development than AAV-based gene therapies.[52] This phase 1 study reported stable FVIII expression using the OSA (ranging from 1.7 IU/dL to 39.9 IU/dL) and an ABR of zero, and the reported TEAEs were related to expected complications of chemotherapy conditioning.[52]

Immune-related toxicity and genotoxicity

AAV-based gene therapy

Immune-related toxicity, particularly hepatotoxicity, is a known AE associated with AAV-based gene therapy. The innate immune response hours after viral vector gene transfer can lead to acute toxicities, with activation of the adaptive immune system, antibody production, and resulting cytotoxic T cell responses.[104] Clinical trials of AAV-based gene therapy have provided key learnings that have allowed cautious progression with other gene therapy approaches.

Immune-mediated rejection is a crucial challenge to human gene therapy.[104] Viral-based vectors trigger a specific host immune response involving a complex interplay between the innate and adaptive immune systems (T cells and B cells),[104] typically with a rise in aminotransaminases and a decline in coagulation factor activity level, which can impact the initial transduction process and affect the long-term durability of transgene expression.[13,34] If a capsid immune response is reported, immunomodulation can be administered to maintain transgene expression and may even be initiated prophylactically prior to treatment[13]; the success of this approach can vary as it is likely to be influenced by recipient- and vector-related features.[13,105] Furthermore, as 30%–60% of patients have pre-existing anti-AAV nAbs from a wild-type infection that reduce or completely block transgene expression, the efficiency of re-administered AAV-based therapy may be limited due to immune memory.[35] Therefore, the identification of total antibodies or anti-AAV nAbs is mandatory in gene therapy trials using rAAV vectors,[12,35] and most AAV-based gene therapy trials define a specific anti-AAV antibody titer threshold as part of their eligibility criteria, essentially excluding a substantial subset of individuals with pre-existing nAbs from participating.[15,49–51] Consequently, multiple regulatory recommendations include the development of concurrent diagnostic tests to screen for pre-existing anti-AAV antibodies.[106–109] Additionally, several physical and pharmacologic methods are being employed to reduce the levels of anti-AAV nAbs and thereby increase the pool of individuals eligible for gene therapy.[110]

As recombinant AAV vectors lack viral genes and do not encode the AAV Rep protein, they cannot efficiently integrate into the host

DNA; however, low-efficiency integration into the host genome in animal models has been reported.[111,112] An incidence of hepatocellular carcinoma after clinical AAV gene therapy for hemophilia B revealed low-frequency AAV vector integration without clonal expansion or dominant integration events, supporting evidence that hepatocellular carcinoma was not due to AAV vector integration.[113]

LV-based gene therapy

In contrast to the requirement for anti-AAV antibody testing with rAAV-based gene therapy, in the *ex vivo* LV-based gene therapy trials under way, capsid seropositivity is not cited in the eligibility criteria; instead, individuals must be free of inhibitors, liver or other organ dysfunction, and diseases such as hepatitis B/C, HIV, and malignancies. Although the prevalence of pre-existing antibodies against vesicular stomatitis virus surface glycoprotein (used to pseudostyle LV) is negligible since it does not infect humans,[29] immune responses targeting the LV or transgene product could still limit the efficacy and safety of the transgene.[29] It is also noteworthy that while AAV vectors induce a relatively weak and transient innate immune response in a low-dose range, LVs induce an adaptive response that limits transgene transduction and may amplify adaptive immunity in preclinical models.[104]

For retroviral-based gene therapies, such as LV-based gene therapies, genotoxicity from gene transfer into progenitor cells may be a potentially greater concern than immune-related toxicity, with semi-random integration into the chromatin of target cells causing clonal expansion potentially leading to malignancy in some cases.[32] Although data indicate that prototypical LVs have lower oncogenic potential than gammaretroviral vectors,[47,114] such events have also been documented in LV trials whereby clonal expansions related to LV insertions within the oncogene were associated with hematologic malignancy.[44] The risk of insertional mutagenesis in LVs is influenced by vector design. For example, self-inactivating LVs (lacking strong promoter-enhancer long terminal repeat sequences) preferentially integrate away from the transcription start sites of actively transcribed genes and have a lower probability of oncogenic selection[45,115]; in contrast, an intact enhancer could drive gene expression of the nearby endogenous gene and clonal expansion, with the potential to lead to malignancy.[44]

Durability of viral vector-based gene therapy

AAV-based gene therapy

The success of gene therapy relies on the initial therapeutic response and a durable long-term response. Data from animal models and early clinical trials suggest *F9* transgene expression using rAAV vectors can be maintained for at least 10 years in adult individuals.[34] After systemic administration of an rAAV8 vector to individuals with severe hemophilia B, stable therapeutic expression of FIX was reported over 8 years, without evidence of late toxicities. Additionally, skeletal muscle expression of FIX protein and RNA was also detected 10 years after AAV-F9 gene transfer in an individual with severe hemophilia B.[116] For FVIII gene therapy, however, a loss of transgene expression over time has been reported.[34,101] The durability of rAAV-based transgene expression can be impacted by several variables that may be nonimmunologic (e.g., cell turnover, cell stress, patient lifestyle factors), immunologic (e.g., innate/adaptive immune response, bystander effect), or an interplay of both.[34] With nonintegrating rAAV vectors, persistence of the therapeutic effect depends on maintenance of circular episomes in nondividing cells; targeting tissues with low cellular turnover may allow episomal DNA to persist for many years, leading to durable transgene expression.[34] However, the use of vectors characterized by host integration (e.g., LV) may allow durable expression of transgenes in tissues with a high cellular turnover.[34]

Another challenge with some AAV-based gene therapy is durability in pediatric patients whose livers continue undergoing rapid cell division; such therapies rely on transgene expression from extrachromosomal episomes not passed to daughter cells. The three approved AAV-based gene therapies, etranacogene dezaparvovec[19] and fidanacogene elaparvovec[36] for hemophilia B and valoctocogene roxaparvovec for severe hemophilia A,[17] are limited to adults and not yet licensed for use in children with hemophilia.

LV-based gene therapy

LVs can transduce dividing and nondividing cells by integrating their genetic cargo into the genome of target cells.[12] This may potentially ensure long-term expression of FVIII or FIX in pediatric patients, thereby overcoming some of the limitations of AAV-based gene therapy.[12] The likelihood of nAbs developing against LVs is low,[29] and LVs can be engineered with a relatively large packaging capacity (~8 kb) suitable for delivering FVIII transgenes.[30] However, as with AAV vectors, innate and adaptive host immune responses to LVs and transgenes may limit the efficacy and safety of the therapy.[29,104] Long-term assessment of *ex vivo*, systemic, and locally administered LV-based gene therapies will fully inform safety and efficacy in the clinic.

Gene editing therapies

Another type of gene therapy, gene editing using CRISPR-Cas9, is in the early stages of clinical investigation for the treatment of moderate to severe hemophilia B, and data are not yet available. A phase 1/2 dose-escalation and confirmation trial of an FIX gene insertion therapy, REGV131-LNP1265, is currently ongoing in patients aged ≥18 years (NCT06379789); the trial is expected to be completed in 2032.[25] REGV131-LNP1265 treatment uses dual LNP and AAV as an effective modular approach for targeted and stable DNA insertion.[25,27] The technology comprises REGV131, which delivers a promoterless DNA template encoding wild-type FIX to hepatocytes using rAAV8, and LNP1265, an LNP delivering Cas9 messenger RNA and a single guide RNA that targets the albumin locus.[27]

Precision genome editing can also be used to engineer B cells, which produce thousands of proteins per cell per second, to produce therapeutic proteins of interest. These engineered B cell medicines are potentially durable, allogeneic, redosable, and administrable without

pre-conditioning. The US Food and Drug Administration granted Orphan Drug Designation to BE-101, a novel engineered B cell medicine being developed for the treatment of hemophilia B, and a phase 1/2 study evaluating BE-101 in adults with severe or moderately severe hemophilia commenced in 2024.[117,118]

Potential immune-related toxicity and genotoxicity in gene editing

The high seroprevalence of nAbs against AAV[35] is a potential immune-related challenge with gene editing, as AAVs are used to deliver the donor DNA. Other potential genotoxicity complications of CRISPR-Cas9 use include off-target events, DNA deletions or other complex rearrangements, and the impact of double-strand DNA breaks on hepatocytes.[12,38,40,119–122] The expression of the CRISPR components is transient, allowing on-target gene editing with potentially reduced risk of off-target effects relative to continuous expression via viral delivery. Although off-target effects have been observed in human cell culture with sustained Cas9 expression,[123] these may be due to assay-specific features such as cell type, expression level, guide sequence, transfection method, and constitutive nuclease expression.[40] Moreover, the frequency of detected off-target effects in *in vivo* models is not statistically distinguishable from background rates of mutation occurring naturally.[124]

Potentially, integration of the AAV vector with associated CRISPR-Cas9 components could be followed by continuous production of the bacterial Cas9 protein and guide RNA.[12] While Cas9 does not appear to be a strong immunogen,[125] some studies have shown prior exposure to Cas9 to be associated with anti-Cas9 protein nAbs and T cell memory, which could potentially limit the effectiveness of CRISPR-Cas9 therapy and present a risk of AEs associated with cytotoxicity or acute inflammation.[126] Subsequent investigations in mice revealed that pre-existing immunity to SaCas9 was accompanied by a cytotoxic T cell response, characterized by hepatocyte apoptosis, loss of recombinant AAV genomes, and complete elimination of genome-edited cells.[126] However, a recent study evaluating CRISPR-Cas9 in hereditary angioedema reported that anti-Cas9 protein antibodies did not negatively impact outcomes, pharmacokinetics, or pharmacodynamics.[94] Furthermore, CRISPR-Cas9 technologies that include Cas9 mRNA (instead of protein)[27] are not impacted by the presence of such antibodies.[94] The clinical translation of CRISPR is yet to be determined; however, current clinical studies have not reported AE outcomes such as off-target effects.

Durability of gene editing

Gene editing may involve precise and accurate functional correction or site-specific integration at the genome level to provide durable and lifelong physiologic gene regulation. In contrast, the lifelong durability of episomal gene expression for FVIII appears to wane over time,[101] and long-term durability of FIX expression is not yet known.

rAAV-based gene therapy has been demonstrated to persist for years in adults who received certain gene therapies, but the consequences of natural cellular turnover on the persistence of episomal AAV and the long-term fate in dividing cells in pediatric recipients is unknown. Gene editing may allow more physiological control of transgene expression than the use of heterologous promoters in current versions of AAV-based gene therapy. Furthermore, as only relatively marginal increases in FIX are needed to maintain hemostatic control and avoid any challenges associated with overexpression, such as thrombosis, understanding and predicting features that could cause variable or supranormal factor levels is critical.

TRANSFORMATIONAL THERAPY FOR HEMOPHILIA B

Considerations for integration of gene therapy in clinical practice

Gene editing technologies, which lag behind rAAV-based gene therapy in clinical testing, stand to gain from discussion around the challenges of delivering advanced gene therapies to individuals with hemophilia. In addition, collection of efficacy and safety data during years of clinical testing and the practical aspects of delivering gene therapies, which require multidisciplinary care and expertise, must be carefully considered.[127] Proposals for gene therapy include the introduction of a certification process for hemophilia centers by the European Hemophilia Network and the exploration of roles and responsibilities within centers so they are better equipped to deliver gene therapy and can improve accessibility.[128] Highly coordinated integrated models (e.g., "hub and spoke") are recommended for the preparation and administration of the gene therapy product, measurement of coagulation and immunologic parameters, and long-term efficacy and safety surveillance (e.g., monitoring joint score and function, and liver health).[128]

Short-term monitoring of recipients of advanced gene therapy is likely to be intensive, and integrated infrastructures may be needed to oversee all aspects of care. Physiotherapists will likely play an essential role in continued joint-health monitoring for recipients of gene therapy. However, the time burden should be minimized for patients by gathering only the essential information.[127]

The practical application of novel gene therapies is nuanced by differences in delivery systems and vector serotypes, transgene modifications, manufacturing, dosing, administration, and approach to follow-up.[129,130] In providing information about gene therapy for hemophilia, physicians should make it clear that research is ongoing and that there are a number of "unknowns," such as the duration of treatment effect and long-term safety profile, including potentially delayed effects.[131] Healthcare providers will be required to educate individuals with hemophilia and their families and caregivers to ensure complete awareness of the risks and benefits of gene therapy before administration.[127] Given the expanding evidence base, physicians and patients need precise, reliable, and up-to-date resources to help explain the risks and benefits of treatment and the eligibility criteria and facilitate shared decision-making.[132,133]

Expansion to the pediatric population

Gene therapy targeting dividing cells may offer long-term, functional, one-time treatment, eliminating the need for regular

exogenous factor infusions in children for whom episomal gene therapy may be ineffective.[34] The nonintegrating feature of AAV limits this approach to long-lived cells in contrast with the integration of LV gene therapy and CRISPR-Cas9, which may enable more durable expression in tissues with higher cell turnover rates.[34] As prophylaxis studies have shown, the earlier hemostasis is maintained, the greater the likelihood of preventing joint damage.[134] Therefore, early corrective treatment of moderate to severe hemophilia in pediatric patients may prevent the long-term sequelae of arthropathy and help avoid a lifetime of injections, pain, inhibitor development, and impaired quality of life.

CONCLUSION

Transformative gene therapies for hemophilia have now been approved, and there are substantial ongoing efforts with different strategies to optimize the safety, efficacy, and feasibility as well as patient eligibility. CRISPR-Cas9-mediated gene editing may offer advantages, including a high degree of efficiency and precision, and broad accessibility, with a high number of eligible patients and the ability to treat the pediatric population. As long-term data are acquired for approved treatments and novel gene therapy techniques complete clinical testing, researchers will better understand the potential efficacy and safety considerations. When available, these data in addition to real-world experience registries may better inform treatment decision-making by patients and their families and caregivers, as well as for researchers, payers, and regulatory bodies involved in gene therapy development.

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AUTHOR CONTRIBUTIONS

Authors contributed equally to conceptualization, creating the initial draft of the manuscript, writing, review, and editing of subsequent drafts.

DECLARATION OF INTERESTS

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