Minireview

Gene therapy for hemophilia: looking beyond factor expression

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Impact Statement

Severe hemophilia patients present with recurrent joint bleeds and consequent progressive arthropathy. For many years, hemophilia management was based on factor replacement, with frequent intravenous infusions of plasma-derived and recombinant factor concentrates. Gene therapy is a paradigm-shifting therapy for hemophilia, with the potential to cure or change the clinical phenotype of a patient with single-dose treatment. This relatively new therapeutic approach has shown to be efficient and mostly durable, but many issues and questions must be addressed before it is widely used in clinical practice. Our publication highlights the main topics about gene therapy efficacy and reviews the recent data about the vector-directed immune response. Currently, extensive clinical and basic investigation on these topics results in ever-changing knowledge, with some answers to old issues but also new questions to be addressed.

Abstract

Hemophilia A (factor VIII [FVIII] deficiency) and hemophilia B (factor IX [FIX] deficiency) are the X-linked recessive bleeding disorders that clinically manifest with recurrent bleeding, predominantly into muscles and joints. In its severe presentation, when factor activity is less than 1% of normal, hemophilia presents with spontaneous musculoskeletal bleeds and may progress to debilitating chronic arthropathy. Management of hemophilia has changed profoundly in the past decades. From on-demand to prophylactic factor concentrate replacement, the treatment goal shifted from controlling bleeds to preventing bleeds and improving quality of life. In this new scenario, gene therapy has arisen as a paradigm-changing therapeutic option, a one-time treatment with the potential to achieve sustained coagulation FVIII or FIX expression even within the normal range. This review discusses the critical impact of adeno-associated virus (AAV) gene transfer in hemophilia care, including the recent clinical outcomes, changes in disease perceptions, and its treatment burden. We also discuss the challenging scenario of the AAV-directed immune response in the clinical setting and potential strategies to improve the long-lasting efficacy of hemophilia gene therapy efficacy.

Keywords: Hemophilia, genetic therapy, blood coagulation factors, factor VIII, factor IX

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Introduction

Hemophilia A (factor VIII [FVIII] deficiency) and hemophilia B (factor IX [FIX] deficiency) are the X-linked recessive bleeding disorders that clinically manifest with recurrent bleeding, predominantly into muscles and joints. In its severe presentation, when factor activity is less than 1% of normal (i.e. <1 IU/dL), hemophilia presents with spontaneous musculoskeletal bleeds and may progress to debilitating chronic arthropathy.[1]

Management of hemophilia has changed profoundly in the past decades. From on-demand to prophylactic factor concentrate replacement, the treatment goal shifted from controlling bleeds to preventing bleeds. Moreover, as prophylaxis evolved, so did the ambition of eradicating chronic joint disease and achieving a virtually normal life expectancy.[2]

Gene therapy has arisen as a paradigm-changing therapeutic option, a one-time treatment with the potential to achieve sustained coagulation factor expression even within the normal range. It feels both old and new – it has been pursued for many years, but only the last decade witnessed the mounting quantity of different clinical trials and substantial clinical results. Even with one gene therapy product already licensed by the European Medicine Association (EMA), the hemophilia community is still working on expanding knowledge and better understanding this life-changing treatment.[3]

In this review, we will discuss the most critical impact of hemophilia gene therapy on patients' daily life, including the potential benefits beyond factor expression. Second, we will describe the challenges imposed by the immune response to adeno-associated virus (AAV) vectors, the primary gene therapy delivery system used by clinical trials for hemophilia. Finally, we will briefly discuss some approaches to

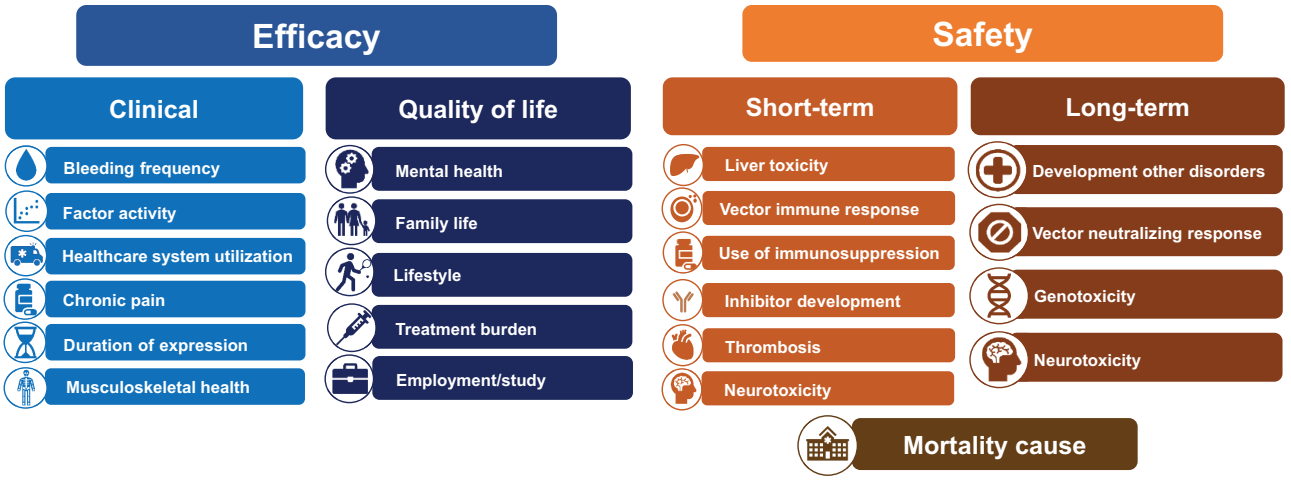

Figure 1. Critical safety and efficacy outcomes to be monitored after adeno-associated virus (AAV)-mediated liver-direct gene therapy for hemophilia. (A color version of this figure is available in the online journal.)

mitigate the anti-AAV immune response and potential strategies to improve the long-lasting efficacy of hemophilia gene therapy efficacy.

Monitoring response beyond factor activity

Since the first report in 2011 of a successful AAV-based liver-directed FIX gene transfer for hemophilia B,[4] several other investigational gene therapy clinical trials for patients with hemophilia A[5–8] and hemophilia B[9–12] have been conducted using the same approach. The knowledge and experience we gained from these clinical trials are helping to design the critical safety and efficacy outcomes to be monitored after hemophilia patients receive the gene transfer (Figure 1).

With growing evidence that treating hemophilia goes way beyond increasing factor activity, multiple stakeholders collaborated to discuss and create the coreHEM project, a set of essential outcomes to evaluate gene therapy for efficacy, safety, effectiveness, and value.[13] This process included patients, patient advocates, clinicians, researchers, government organizations, payers, and companies with ongoing hemophilia gene therapy programs.

coreHEM used a modified Delphi consensus process to create and rate a list of candidate outcomes. An interesting detail of this collaborative task resides in the fact that outcomes that were eliminated by the group but considered important by the patients were reproposed for voting by the group. This strategy ensured that significant outcomes for the patients were not rejected before a deeper discussion among the whole group.

This initiative produced a core outcome set, with the most critical outcomes for the proper evaluation of gene therapy. Factor expression and frequency of bleeds continue to be important parameters but are complemented by the duration of expression, chronic pain, mental health status, and utilization of the health-care system.[13,14]

Coagulation factor activity, duration of expression, and bleeding frequency

Coagulation factor activity has been the primary outcome in most hemophilia gene therapy trials.[4,8–10] It is a logical choice, and there are quite a few reasons to support it. First, factor activity is a hard outcome, meaning it is not prone to subjectivity from the investigator or the patient. It allows a more objective evaluation of efficacy than other outcomes like patient-reported bleeding rates, a frequently used outcome for efficacy evaluation in hemophilia. In addition, it is widely known that significant changes in hemophilia phenotype are seen with small increments in factor activity, meaning that even low increments in factor activity may have a substantial impact on bleeding frequency and the burden of both disease and treatment.

As it is the case for many scenarios in the hemostasis laboratory, the choice of employed methodologies matters for assessing the transgenic FVIII and FIX expression and the two available assays for factor activity were shown to yield different results. For hemophilia A gene therapy, one-stage assay (OSA) was initially the chosen methodology to evaluate postgene transfer FVIII activity since it is more available in clinical practice due to its lower cost. Also, recombinant B-domain-deleted factor VIII (r-BDD-FVIII) traditionally has shown lower activity in OSA than chromogenic-substrate assay (CSA).[15–17] Yet, the opposite was seen with transgene-produced BDD-FVIII, with BioMarin's valoctocogene roxaparvovec data showing OSA results being 1.6 times higher than CSA results, a finding that was consistently observed in other studies.[5,6,18] This unexpected discrepancy could be explained by an apparent faster activated factor X (FXa) production by transgene-produced BDD-FVIII.[19] Currently, CSA is frequently used to report FVIII activity after gene therapy. Yet, a correlation analysis of reported joint bleeds and FVIII activity suggests that OSA is better at predicting bleeding risk, particularly for FVIII activity under 15 IU/dL.[19,20]

For the recent hemophilia B gene therapy clinical trials, the transgene used has been the FIX-Padua, a gain-of-function FIX variant due to the substitution of arginine to leucine at position 338, first identified in a young man with venous thrombosis with FIX activity 700%.[21] This FIXR338L variant results in a protein with approximately eightfold higher specific activity, which represents an advantage for gene therapy, achieving therapeutic levels of FIX with a lower AAV vector dose.[9] However, once again, a notable measurement

discrepancy between the methodologies used to measure the FIX-Padua transgene activity is observed. The OSA FIX levels are two- to three-fold higher than the CSA, which is likely caused by the insufficient factor X content in the chromogenic reagent mix that influences the higher specific activity of the FIX-Padua variant, when measured in this assay.[22,23]

As for the duration of expression, the longest-running clinical trial reported sustained FIX expression for more than 10 years of follow-up.[24] For hemophilia A, the largest phase 3 clinical trial recently published its results after 134 patients completed a minimum follow-up of one year, with a median chromogenic-substrate FVIII activity level of 23.9 IU/dL at the end of the first year postinfusion.[8] However, the two-year follow-up of these patients, including the analysis of 17 patients with a three-year cutoff, shows a decline in FVIII expression over time, although with persistent clinical benefit.[25] These findings are consistent with the results from the phase 1/2 trial with the same therapy, which have shown a similar decline in FVIII activity after a five-year observation period of 13 subjects.[7] Contrastingly, the phase 1/2 SPK-8011 study reported stable FVIII levels in 16 of the 18 dosed subjects, with 12 patients (66.7%) followed over two years.[6]

The proper identification of new bleeding events in the gene therapy setting may require a learning curve for both health-care professionals and patients. For many years, patients have been advised to treat any episode of acute joint pain or to worsen chronic joint pain as a new bleed. Yet, there are other possible causes for pain in a patient with advanced arthropathy and chronic synovitis. When there is a worsening of chronic pain but no evident hemarthrosis (no edema, decreased range of motion, or increased joint temperature), point-of-care ultrasound is a helpful tool that allows proper identification of new joint bleeds, especially in patients with some factor expression. It is a non-invasive exam that allows comparison with baseline images – an important feature in the context of chronic arthropathy – and is useful in the evaluation of treated bleeds.[26] However, it could add to the treatment burden since it still requires a visit to the hemophilia center whenever there is a suspected bleed.

Once sustained factor expression was proved to be achievable with gene therapy, the dream of "curing" hemophilia seemed closer than ever, setting high expectations from the whole hemophilia community. Still, it is important to stress that even patients who reach persistently low factor activities do have a significant benefit, as data from clinical trials show that most subjects remain off prophylaxis and with a significant decrease in treated bleeds.[7,25]

The lack of predictors for response and durability is currently one of the major challenges for gene therapy. Another significant challenge is to understand the influence of vector-directed immune response in short- and long-term efficacy. This matter will be further discussed in a later section of this article, as there are conflicting data regarding this association.

Musculoskeletal health and chronic pain

As therapeutic alternatives' efficacy and availability evolved in the past decades, the aim of the treatment for severe hemophilia patients has shifted from improving life expectancy to enhancing the quality of life and musculoskeletal (MSK) health. Prophylaxis has significantly impacted these outcomes and, in the past years, has evolved from simply aiming a factor activity trough level above 1% to individualized dosing schemes based on the patient's lifestyle, joint health status, and pharmacokinetic profile. However, even when adherent to high-dose prophylaxis and with no reported bleeds, some patients present progressive deterioration of joint health. Manco-Johnson *et al*.[27] have shown joint damage in magnetic resonance imaging data of 20% of patients under prophylaxis and reportedly asymptomatic. In this scenario, subclinical unrecognized bleeds have been deemed responsible for the progression of joint disease and prove that better therapeutic options should be pursued.[28] Data concerning the impact of gene therapy in MSK health are still not available but are highly expected. Studies with radiographic joint evaluation in gene therapy patients (with magnetic resonance imaging or even ultrasound) might be very informative.

Increasing factor activity trough levels could be an alternative to prevent subclinical bleeds. Data from Den Uijl *et al*.[29] have shown that only factor levels of 15% are enough to avoid the risk of spontaneous bleeding, a difficult target to achieve with regular replacement therapies. The latest edition of the World Federation of Haemophilia (WFH) guideline suggests of minimum trough level of 3–5%,[2] which, despite being lower, is still a considerable challenge for many people with hemophilia and hemophilia caregivers around the world. Consequently, data on musculoskeletal health from patients treated with gene therapy are highly expected. This is the first treatment that could provide a stable factor expression of over 15% and even more than 40% in some patients.

Chronic pain is generally defined as pain that persists longer than three months[30] and can be a lifelong burden for many individuals with severe hemophilia, being reported by over 50% of this population.[31] It could be continuous or intermittent, and its intensity may vary over time. It is mainly associated with chronic arthropathy, and although very prevalent, no consensus or guidelines for the proper management of chronic pain in hemophilia patients are currently available. Simple analgesics (such as paracetamol) and non-steroid anti-inflammatory drugs are the most used agents, but opioids are also prescribed in some situations.[32] Managing pain in the scenario of liver-directed gene transfer is challenging, as the chronic use of potentially hepatotoxic drugs is a significant concern for factor expression durability.

It is already known that tertiary prophylaxis with factor replacement improves function, quality of life, activity, and pain, although it did not impact joint structure assessment by magnetic resonance imaging (MRI).[33] Recently, Kiialainen *et al*.[34] reported a clinically relevant improvement in Hemophilia Joint Health Score (HJHS) in hemophilia A patients without inhibitors under emicizumab prophylaxis. This is the first evaluation of the impact of a "stable hemostasis" on joint health, without peak and trough levels of regular factor replacement and it opens the debate as to whether patients reaching stable factor levels after gene therapy will have similar improvement. As chronic arthropathy significantly affects other vital outcomes for hemophilia (such as chronic pain, quality of life, absenteeism, and

others), improving joint health could have a long and maybe unknown list of additional benefits.

Health-related quality of life

Health is a complex biopsychosocial construct, and it is not interpreted solely as the "absence of disease."[35] Assessing the impact of health on one's quality of life is still a challenging mission, as most of the current questionnaires in hemophilia do not appear to adequately address the personal and subjective nature of an individual's quality of life.

Haemo-QoL is a hemophilia-specific tool, validated to assess quality of life in children, adolescents, and adults with hemophilia. The adult version, Haemo-QoL-A, comprises six domains (consequences of bleeding, emotional impact, physical functioning, role functioning, treatment concern, and worry) and is widely used to detect quality of life changes following standard therapy in hemophilia A, being recently validated for gene therapy as well.[36] Data on quality of life after gene therapy for hemophilia A and B have been assessed and reported with Haemo-QoL-A so far.[7,37]

A major issue when evaluating quality of life is that most questionnaires are formative measures, meaning that they usually account values of groups and not the values of the individual being questioned. In addition, Haemo-QoL-A is often reported as a number and analyzed as a continuous variable, when a single overall number may not be appropriate when expressing quality of life. Yet, analyzing and comparing qualitative data are challenging, especially concerning such a bias-prone and subjective outcome such as quality of life.

Despite being frequently discussed in any chronic disease scenario, quality of life was not included as a critical outcome in the evaluation of gene therapy by the coreHEM initiative.[13]

Mental health

While novel therapies and improved care have brought the possibility of average life expectancy to hemophilia patients, much work must be faced until they are also expected to have a "normal" quality of life. Hemophilia patients have a compromised quality of life compared to the general population,[38] and mental health status is a significant determinant of this finding.

Hemophilia imposes many harsh challenges for both patients and their families from early childhood, with an unequivocal impact on every aspect of life, such as employment, productivity, personal relationships, and emotional well-being, among others. In a recently published meta-analysis, hemophilia patients were found to have 2.6 times higher risk of depression, 1.74 times more increased risk of anxiety, and 3.48 times higher risk of depression associated with anxiety.[39] The higher prevalence of mental health disorders in hemophilia patients is both a consequence and a cause of decreased quality of life, which makes mental health status a critical outcome for transformative therapies such as gene therapy.

Assessing mental health status in gene therapy is a considerable challenge, as it is influenced by the patient's ability to cope with hemophilia, their expectations toward the treatment itself, and the life-changing possibility of a cure. Therefore, proper hemophilia-specific tools must be developed and validated to be used in this particular scenario. The challenge lies in finding a proper way to show therapy-driven effects on mental health status.

Utilization of health-care system and indirect treatment costs

Hemophilia care places a considerable economic burden on people with hemophilia, their families and caregivers, and the health-care system. Prophylaxis alone determines a significant part of the expenses, with a projected yearly cost of approximately US$700–US$750,000, depending on the chosen type of factor concentrate.[40] Although the cost of factor concentrates is responsible for over 80% of health-care costs of people with hemophilia,[41,42] other costs should not be underestimated. Hemophilia patients have higher rates of office visits, hospitalizations, emergency room visits, medical procedures, and laboratory tests, incurring an even higher treatment cost altogether.[43,44]

A few studies have investigated the cost-effectiveness of gene therapy in different scenarios of hemophilia, with concordant results. Considering a lifelong period starting at the age of 18 years, AAV-based gene therapy with FIX-Padua was considered more cost-effective than prophylaxis for hemophilia B in 92% of the simulations made by Bolous *et al*.,[45] for both standard and extended half-life factor concentrates. In hemophilia A, gene therapy has shown to be cost-saving and more effective than standard prophylaxis, either considering a 10-year period or a lifelong period.[46,47] These results should be interpreted with caution, since FVIII levels beyond 6 years postgene therapy infusion are still uncertain.[48] Assessment of the cost-effectiveness of gene therapy in comparison with non-replacement therapies is not available yet. Still, it should be performed, as well as the analysis of gene therapy in a resource-limited scenario, as a one-time treatment with the potential to provide sustained FVIII or FIX expression.

AAV-driven immunogenicity and gene therapy efficacy – an unclear association

Vector-directed immunogenicity is one of the major setbacks in AAV-based gene transfer, as both innate and adaptive immune responses may significantly impact short-term response and efficacy.

Innate immune response

Innate immunity is the earliest response upon exposure to a pathogen. Since it is not antigen-specific, it is an early and fast response and does not result in immunologic memory. It is mainly triggered by pattern-recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs). PRRs can recognize viral nucleic acids, membrane glycoproteins, and chemical messengers, activating the nuclear factor-κB (NF-κB), and interferon (IFN)-γ regulatory transcription factors. Once activated, these pathways create a pro-inflammatory state, triggering the adaptive immune response.[49]

For AAV vector, innate immunity is activated by the viral capsid, DNA components as well as vector contaminants, but

the intensity of response may be augmented when there is a high content of empty capsids or cytosine–guanine dinucleotide (CpG) motifs.[49] In animal models, it has been shown that CpG motifs may activate Toll-like receptor 9 (TLR9), activating innate and, consequently, adaptive immune responses.[50] In addition, it was observed that AAV transduction leads to the formation of intermediate double-stranded RNA in the cytosol of transduced hepatocytes, which could activate innate immunity by the cytosolic RNA sensors.[51]

Recently, data from the BAX335 gene therapy for hemophilia B program have brought some light to the real impact of innate immunity in the clinical setting. BAX335 is an AAV8-based FIX-Padua gene therapy, and specifically, because of codon optimization, its transgene has a five-time higher CpG density (19 CpG motifs for FIX-wild type and FIX-Padua cDNA sequences vs 99 CpG motifs for codon-optimized FIX-Padua cDNA). Seven of the eight patients dosed in this program did not express sustained therapeutic FIX activity. The only subject who presented long-term FIX expression had a heterozygous missense polymorphism in the interleukin-6 receptor (*IL-6R*) gene, specifically in the IL-6-binding domain. The authors hypothesized that the resultant impaired IL-6 responsiveness could explain how BAX335 could evade immune detection and achieve persistent FIX expression in this single participant.[11] Although these results suggest that high CpG content might be associated with steroid-unresponsive immune response, data from SPK-9001, a vector with zero CpG in the FIX open reading frame, reported 2 out of 10 subjects still developed cellular immune response.[52]

Adaptive immune response

The adaptive immune response includes both antibody-mediated and cellular immunity and is an important topic before and after vector infusion in AAV-based gene transfer.

Antibody-mediated immune response. In the predosing AAV vector gene transfer scenario, pre-existing anti-AAV neutralizing antibodies have been a primary concern for patient eligibility. The wild-type AAV is a naturally occurring virus not associated with diseases, but its infection seems to be very common, depending on the serotype and geographic region. Recently, a seroprevalence study for different AAV serotypes (AAV2, AAV5, AAV6, AAV8, and AAVrh10) was conducted with male subjects with hemophilia A. The global seroprevalence for all serotypes ranged from 34.8% to 58.5%, but greater differences were observed among the participating countries. For example, while subjects from South Africa presented with a seropositivity rate for anti-AAV2 of 94.6%, its prevalence in Japanese subjects was only 43.4%. This gap was also observed for other AAV serotypes as well. However, for AAV5, one of the common vector serotypes with liver tropism used in hemophilia gene therapy programs, the global average seropositivity rates in hemophilia A patients included in this study were 29.7%, the lowest seroprevalence in this population. Results also showed that anti-AAV prevalence increased with age for all serotypes.[53]

Evidence regarding the real impact of anti-AAV vector antibodies on gene transfer efficacy is conflicting. The earliest gene therapy trials for hemophilia B have shown that pre-existing anti-AAV neutralizing antibody (NAb) may modulate transduction, depending on the infused vector dose.[54]

Most of the gene therapy clinical trials had the exclusion of patients with anti-AAV antibodies. However, among these very same trials, it is not well-defined how to determine the presence or absence of these antibodies, as the methodologies used differ from study to study. Some trials considered an eligibility criterion the absence of total anti-AAV5 capsid antibodies.[5,8,55] Other studies used *in vitro* techniques that evaluate the percentage of transduction reduction with an AAV reporter vector of the serotype of interest, carrying luciferase, or green fluorescent protein to demonstrate the presence of anti-AAV Nab.[56] This reporter vector is incubated with serially diluted samples of patient serum or plasma, followed by addition and incubation with cell culture (usually HEK293 cells). The expression of the reporter protein is then measured – the mean fluorescence intensity (MFI) correlates to the multiplicity of infection (MOI) linearly. The titer is considered the highest dilution that reduces vector transduction by at least 50%. Besides chosen methodology and reagents, the purity of the vector preparation and the presence of inactive viral particles may also influence anti-AAV NAb titer. Considering this complex scenario, Weber has proposed that the results for anti-AAV NAb be reported as the number of AAV particles neutralized per µL of serum/plasma and not as titers.[57] This change would allow comparison between results from different laboratory techniques and would comprehend inhibition by other factors in the serum (non-antibody inhibition). *In vivo* assays are also available and known to be more sensitive but are more expensive and not applicable to large populations.

In contrast with most of the initial studies that excluded anti-AAV Nab-positive patients, there are now ongoing trials on gene therapy for anti-AAV NAb-positive patients. The latest data from the phase 3 HOPE-B hemophilia B program with etranacogene dezaparvovec (AAV5-Padua hFIX variant; AMT-061; NCT03569891; UniQure, CSL Behring, King of Prussia, PA, USA) have demonstrated efficient AAV5 transduction in subjects with pre-existing anti-AAV5 Nab. Among the 54 hemophilia B enrolled in this study, only one, with an anti-AAV5 NAb titer of 3212.3, did not respond. The other 53 patients demonstrated sustained FIX expression until 18 months of follow-up, even with pre-existing anti-AAV5 NAb up to a titer of 700.[58] In addition, BioMarin is now recruiting for a phase 1/2 gene therapy clinical trial designed specifically for severe hemophilia A patients with anti-AAV5 antibodies (NCT03520712).

Cellular immune response. In the postinfusion setting, B- and T-cell responses are part of the adaptive immune system.

Interestingly not anticipated by animal model trials, cases of transaminitis and loss of transgene factor expression were seen in human subjects across multiple trials. This response seems to be dose-dependent, and lower vector doses were associated with a mild immune response usually manageable with immunosuppression with corticosteroids. Manno *et al*.[54] reported a temporal relationship between the decline of FIX expression, transaminitis, and detection of IFN-γ secretion by enzyme-linked immune absorbent spot assay (ELISpot)

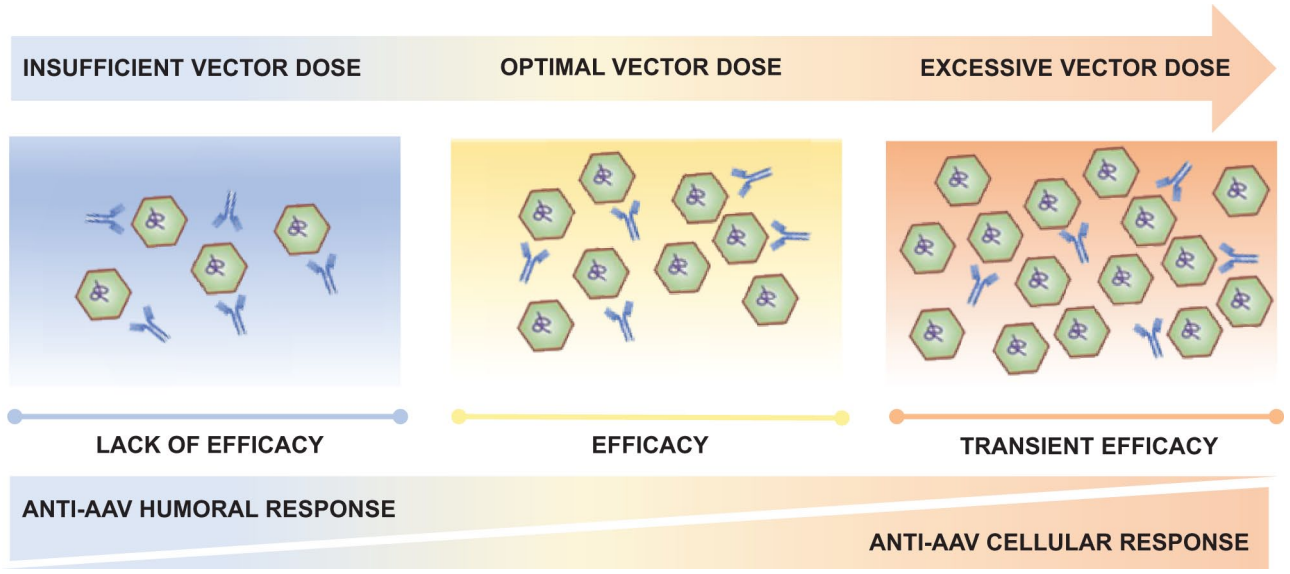

Figure 2. Adeno-associated virus (AAV) vector dose, humoral and cellular immune responses, and efficacy in gene therapy for hemophilia. (A color version of this figure is available in the online journal.)

in their AAV-based hemophilia B gene therapy trial, consolidating the initial hypothesis that T-cell mediated immunity directed to transduced hepatocytes, which transiently expressed vector capsid molecules and were destroyed by activated CD8+ effector T-cells. Yet, this association was not observed in some later trials.[11,55] This disparity could be partially explained by some confounding factors, mainly regarding the methodology used to assess T-cell activity. Evaluation of vector-directed immunity is usually made by ELISpot, a methodology with limited reading capacity that usually evaluates a single cytokine (IFN-γ, IL-5).[59] In addition, most studies use peripheral blood mononuclear cells (PBMCs) for these evaluations, which may not represent the T-cell population that resides in the liver in a proper manner. Also, as most studies start immunosuppression as soon as there is any increase in alanine transaminase (ALT), it is difficult to assess how much interference immunosuppressive therapy could have on the aforementioned assays.

In addition, it is important to notice a significant relationship between AAV vector dose and predicted gene therapy outcome. Lower vector doses are more likely to be neutralized by anti-AAV antibodies, resulting in reduced transduction efficacy. Higher AAV vector doses overcome this limitation, leading to therapeutic efficacy. However, it increases the risk of immune-mediated clearance of transduced target cells resulting in a loss of transgene expression (Figure 2).

Strategies to enhance gene therapy efficacy

Some strategies to enhance AAV-based gene therapy efficacy are summarized in Figure 3.

Improving AAV-mediated gene transfer

AAV is a mainly non-integrating vector, as it delivers the therapeutic transgene as an episome. Only a minimal integration may occur. However, a recent mouse model study showed an integration rate of up to 3% in humanized hepatocyte genome.[60]

The attribute as a mainly non-integrating vector is a desired feature for viral vectors, as it reduces the risk of genotoxicity associated with integration on the wrong site. Yet, this approach may have its toll on durability since only one of the daughter cells will receive the therapeutic episome after cell division. As time goes by, a dilutional effect may tamper with efficacy itself and the duration of transgene expression. An integrating approach, with the use of different viral vectors, could be an interesting alternative to increase the durability of gene transfer. Another strategy is the use of promoters that are stronger and more specific, to limit expression in non-target tissues and enhance gene transfer to the target cell.

The use of an optimal vector dose is critical to a successful transfer – small doses may be insufficient for an adequate gene transfer and may result in neutralization by anti-AAV antibodies, in individuals with pre-existing anti-AAV NAb. However, a very high vector dose could lead to a loss of efficacy after triggering an AAV-directed T-cell response.

Removal of pre-existing anti-AAV antibodies is an approach as challenging, as it is desired, particularly to permit the successful re-dosing of the AAV vector. Plasmapheresis and immunoadsorption have been employed in some studies in both preclinical and clinical settings.[61,62] Recently, imlifidase (IdeS), an IgG-degrading enzyme under investigation for solid organ transplantation, successfully enabled gene transfer in non-human primates with anti-AAV Nab.[63]

Increasing expression efficiency

Codon optimization is a gene engineering process that uses synonymous codon substitutions to enable higher protein expression.[64] In hemophilia A gene transfer, it has reportedly increased FVIII secretion by 30% in transfected cells.[65] Consequently, codon-optimized B domain-deleted FVIII gene sequences are employed in all ongoing hemophilia A gene transfer clinical trials.[4,8,18] The use of hyperactive variants, such as PIX-Padua, a gain-of-function mutation in *F9*, has been shown to lead to higher FIX levels in hemophilia B

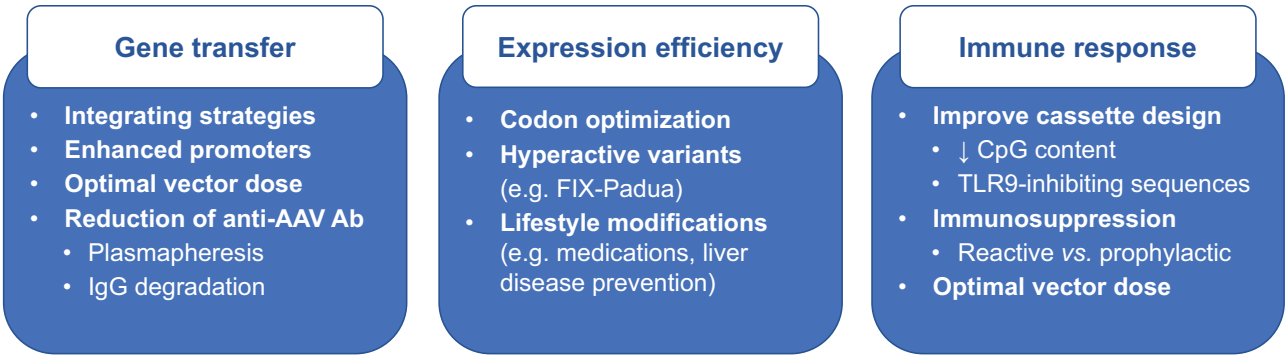

Figure 3. Strategies to enhance efficacy in gene therapy for hemophilia. (A color version of this figure is available in the online journal.)
AAV: adeno-associated virus; IgG: immunoglobulin G; FIX: factor IX; TLR-9: Toll-like receptor 9.

gene therapy trials, with persistent expression in an ongoing trial.[58,66]

Lifestyle modifications to maintain liver health are also recommended to patients undergoing gene therapy for hemophilia, with the intention of reducing the impact of hepatotoxicity and liver stress in gene therapy efficacy. Therefore, patients should be advised to abstain from alcohol consumption, both in short and long term, as it is still unknown what a safe alcohol intake is in liver-directed gene therapy scenario. Also, patients are also instructed to avoid any potentially hepatotoxic medications, whenever possible, and are encouraged to pursue a healthy diet and regular physical exercise, in both pre- and post-gene therapy scenarios.

Mitigating immune response

If the impact on the transduction efficacy, excluding patients with pre-existing anti-AAV antibody, still needs to be evaluated, immunosuppression is the strategy used to minimize the AAV-directed cellular immune response. Among the immunosuppressants used, corticosteroids were the first agents to be used. Still the primary choice in most ongoing trials, they have a broad inhibitory effect on both innate and adaptive immunity by reducing pro-inflammatory cytokines and T- and B-cell proliferation. Initial trials have shown that courses of oral prednisone were enough to block the T-cell-driven hepatocyte injury and rescue coagulation factor expression.[4] Yet, this approach was not successful in all patients, and alternative immunosuppressants were needed in some cases.[12] Data on alternative immunosuppressive agents are even more scarce, a challenging scenario to currently draw any conclusions.

Other alternatives lie in the engineering and manufacture of the vector, either by making it less immunogenic (with decreased CpG content, contaminants, or empty capsids) or by enhancing its transduction capacity in order to reduce the therapeutic dose.[57] Novel recombinant vectors could also be a solution, but the high cross-reactivity between AAV serotypes could still be an obstacle.

Expanding target population for hemophilia gene therapy

Inhibitor patients

The development of neutralizing alloantibodies against FVIII or FIX (inhibitors) still is the most challenging and significant complication of coagulation factor replacement therapy. Inhibitors develop in approximately 30% of severe hemophilia A and 3% of severe hemophilia B patients[67] and when present, completely impair factor replacement hemostatic efficacy. Consequently, the presence of inhibitor has been historically associated with an arduous scenario of increased physical and psychosocial morbidity and higher mortality.[67] The recent development and licensing of emicizumab, a bispecific monoclonal antibody with FVIII-mimicking activity, has changed the landscape of inhibitors in hemophilia A and ignited the discussion of whether tolerance induction should still be pursued in those patients.[68,69] Yet, inhibitors patients with hemophilia B still lack an optimal management, with many relying only on recombinant activated factor VII as their sole available hemostatic agent.

Initially, the presence of active or prior inhibitors was a consistent exclusion criterion through every hemophilia gene therapy trial. However, data from hemophilia A and hemophilia B canine models have shown that gene therapy may have a dual therapeutic effect in inhibitors patients, first leading to FVIII/FIX tolerance and later providing FVIII/FIX expression.[70–72] Currently, two ongoing AAV-mediated gene transfer trials are recruiting patients with hemophilia A and current or past FVIII inhibitors (NCT03734588 and NCT04684940) and their highly expected results may open a new path in inhibitor management for hemophilia A patients.

Children and adolescents

So far, children and adolescents under 18 years of age have been excluded from all gene therapy trials for hemophilia. Yet, the benefits of having early sustained factor levels on MSK health and the other innumerous aspects of hemophilia care make gene therapy an attractive solution for young patients. Other favorable aspects of treating young patients are their tolerable immune system and their low AAV NAb titers.[73]

Since AAV vectors are predominantly non-integrating, the major challenge in treating young patients with AAV-based liver-directed gene transfer is the potentially dilutional effect from hepatocytes proliferation during liver growth. Integrating approaches, such as lentiviral vectors, could facilitate treatment in replicating cells and be the solution to enable younger hemophilia patients to be treated with gene therapy. The advantage of durable factor expression contrasts with safety concerns around insertional oncogenesis.

Although preclinical and clinical data suggest that lentiviral vectors integration is unlikely to result in insertional mutagenesis,[74] three patients were diagnosed with myelodysplastic syndrome up to 7.5 years after treatment with elivaldogene autotemcel (Skysona®, bluebird bio, Somerville, MA, USA) for cerebral adrenoleukodystrophy.[75] Still, in 2022, US Food and Drug administration (FDA) granted approval for Skysona® for patients aged 4–17 years, along a boxed warning for hematological malignancies.[75] Previously, FDA also approved Zynteglo® (bluebird bio), a lentivirus-based gene therapy for beta-thalassemia for patients over four years of age.[76]

Conclusions

Gene therapy is a promising life-changing treatment for severe hemophilia patients, with the first product recently licensed for use in the European Union.[3] So far, some old issues have been answered, but new questions have risen as more and more patients are dosed in clinical trials, and the hemophilia community is looking forward to the inclusion of individuals with anti-AAV neutralizing antibodies and individuals with inhibitors against FVIII or FIX on the coming years.

AUTHORS' CONTRIBUTIONS

GGY-H and MCO co-wrote the manuscript. The authors have read and agreed to the published version of the manuscript.

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