Hemophilia Gene Therapy: Ready for Prime Time?

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Hemophilia A and B are congenital, X-linked bleeding disorders caused by mutations in the genes encoding for the blood clotting factor VIII (FVIII) or factor IX (FIX), respectively. Since the beginning of gene therapy, hemophilia has been considered an attractive disease target that served as a trailblazer for the field at large. Different technologies have been explored to efficiently and safely deliver the therapeutic *FVIII* and *FIX* genes into the patients' cells. Currently, the most promising vectors for hemophilia gene therapy are adeno-associated viral vectors (AAVs) and lentiviral vectors. More recently, gene editing approaches based on designer nucleases or CRISPR/Cas, have also been considered to minimize risks associated with random vector integration and insertional mutagenesis though off-target issues would have to be carefully and comprehensively assessed. In the past two decades, several phase 1 hemophilia gene therapy clinical trials have been initiated with varying success. In particular, the early gene therapy clinical trials in hemophilia B patients based on AAV showed either transient or subtherapeutic clotting factor expression levels. This could be ascribed, at least in part, to suboptimal vector design and/or inadvertent immune consequences triggering hepatic inflammation. Hence, there was an unmet need to further increase vector safety and efficacy in future trials, preferably by using lower vector doses. It is particularly encouraging that sustained therapeutic FVIII and FIX expression levels have recently been attained after gene therapy in patients with severe hemophilia paving the way towards pivotal trials and commercialization. Nevertheless, transient liver toxicity still occurs and the use of transient immunosuppression was still required to curtail inadvertent immune responses, especially at high vector doses. To further boost clotting factor expression levels, codon-usage optimized synthetic *FVIII* or *FIX* transgenes have been employed. Alternatively, we and others have shown that the incorporation of hyperactive gain-of-function R338L mutation in the *FIX* gene substantially increased the overall efficacy. It is inevitable that the continued improvements in vector engineering and new insights in the vector–patient interactions will further benefit the development of a safe and effective cure for hemophilia A and B.

Keywords: factor IX, factor VIII, coagulation, hemophilia, AAV, lentiviral, CRISPR, ZFN, factor IX Padua

INTRODUCTION

HEMOPHILIA A AND B are congenital X-linked bleeding disorders caused by mutations of genes encoding the blood clotting factor VIII (FVIII) or factor IX (FIX), respectively. Consequently, these disorders are characterized by a bleeding disorder due to a deficiency in the corresponding clotting factors. Patients often suffer from recurrent bleeds and chronic joint damage resulting in a crippling arthropathy. The bleeding could even be fatal in cases of intracranial hemorrhage. According to the

World Federation of Hemophilia, the diseases affect an estimated 400,000 individuals worldwide,¹ of which 80–85% are affected by hemophilia A and the remaining 15% by hemophilia B. Current treatment consists of protein substitution therapy (PST) with recombinant or plasma-derived clotting factors. Although PST improved the patient's quality of life and significantly prolonged life expectancy, it has several drawbacks. First, this treatment is noncurative, implying that the patient remains at risk of developing bleeding episodes

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and/or chronic joint damage. The second constraint pertains to the clotting factors' short half-lives, requiring frequent treatment with prophylaxis or on demand using relatively large doses of FVIII or FIX. Finally, some patients develop neutralizing antibodies (clinically defined as inhibitors) against the injected FVIII or FIX proteins that render further PST ineffective. Several next-generation protein-based therapeutics have recently been developed that prolong the half-life of the clotting factors, for instance by pegylation or fusion with albumin and immunoglobulin domains.² Consequently, these second-generation products require less frequent administration and represent a new benchmark for all emerging products to treat hemophilia patients, including gene therapy. Given the intrinsic limitations of PST, there is still a need to develop a long-term cure for hemophilia via gene therapy.

The current review celebrates the 25th anniversary of the European Society of Gene and Cell Therapy. Our own personal journey in the field of gene therapy for hemophilia coincidentally also started exactly 25 years ago. This allowed us to witness firsthand the exciting developments in gene therapy for hemophilia that had an impact on so many other diseases since then. In this review, we will highlight the most relevant and most recent developments in the field of gene therapy for hemophilia A and B. We will also discuss some of the outstanding questions and remaining challenges that would still need to be addressed. It is beyond the scope of the current review to provide a comprehensive description of all of the possible strategies and animal studies that have been published relating to hemophilia gene therapy in the past 30 years, which have been covered by other earlier reviews on the subject.^{3–5} Instead, we will focus on highlighting a few key concepts with specific emphasis clinical translation.

GENE THERAPY

The main objective of developing gene therapy for hemophilia is to achieve prolonged high-level expression of clotting factors and stably correct the bleeding disease. Essentially, there are two distinct approaches to achieve this objective. The first approach is *FVIII* or *FIX* gene delivery to long-lived post mitotic cells, such as hepatocytes or skeletal muscle cells. In this case, the therapeutic genes should either integrate into the target cell genome or persist as episomal DNA. Alternatively, *FVIII* or *FIX* genes could be delivered to stem/progenitor cells, such as hematopoietic stem cells (HSCs), using integrating (viral or nonviral) vectors in

order to achieve long-lasting expression through their progeny. Designing the optimal *FVIII* and *FIX* expression cassette and delivering these constructs into the appropriate target cells in a safe and efficient manner will be essential toward achieving a cure for hemophilia. Ideally, gene therapy should not result in the induction of inhibitors or should enable clearance of pre-existing inhibitors through induction of FVIII- or FIX-specific immune tolerance, which has been accomplished in preclinical studies.^{6,7} The ability to induce immune tolerance to FVIII or FIX after gene therapy varies depending on several confounding variables, including the transgene product, the vector design, the target cells, and the underlying mutation of the endogenous *FVIII* or *FIX* gene.

Designing optimal *FVIII* and *FIX* expression cassettes

As with any gene therapy approach, it is critically important to express high levels of the therapeutic protein using the lowest possible vector dose to minimize untoward vector-associated immune responses or toxicity. This has been particularly challenging since there are several bottlenecks at the transcriptional, translational and post-translational level, especially for FVIII.⁸ It is therefore necessary to overcome each of these limiting steps using rationally designed transgenes and expression constructs. Incremental improvements at the transcriptional, translational, and/or post-translational levels may ultimately translate into significant dose benefits in clinical trials in hemophilia patients. All of the gene therapy approaches rely on the use of *FVIII* and *FIX* cDNAs instead of the full-length transgenes *per se*. Nevertheless, in many cases, introns were incorporated in the constructs^{9,10} to further boost expression levels that act possibly, at least in part, by facilitating extra-nuclear transport of the cognate transcripts.

The endogenous *FVIII* and *FIX* promoters are not particularly robust and are therefore not ideally suited for gene therapy for hemophilia. Instead, to boost the *FVIII* and *FIX* mRNA levels, we and others have explored the use of robust tissue-specific heterologous promoters, such as α 1-antitrypsin or transthyretin.^{10–12} These promoters could be combined with other regulatory elements (*e.g.*, HCR, enhancers) to further boost expression.¹¹ More recently, we have developed a novel approach to identify robust human hepatocyte-specific *cis*-regulatory modules (CRMs) using genome-wide computational strategies instead of the conventional trial-and-error approaches. The use of these CRMs, in conjunction with existing promoters, resulted in relatively robust increases in *FIX* or

FVIII gene expression when used in the context of different gene therapy vectors.^{13–15} It is reassuring that these CRMs increased expression of heterologous transgenes, including *FVIII* and *FIX*, without increasing the risk of insertional oncogenesis of integrating vectors, even in highly sensitive tumor-prone mouse models.¹⁵ Alternatively, synthetic transcriptional enhancers were generated *de novo* by random ligation of synthetic oligonucleotides coding for binding sites of hepatic transcription factors and *in vitro* screening in hepatic cell lines.¹⁶ The specificity of the promoter and regulatory elements can have important consequences for the immune response directed against the transgene products^{16,17} (see below).

More than 20 years ago, we pioneered the use of codon-optimization of clotting factor genes in attempt to increase their expression.⁹ Since then, we and others have continued to develop codon-optimized *FVIII* and *FIX* transgenes^{10,13,18,19} that resulted in higher levels of protein expression for the same amount of mRNA. One of the main advantages of codon optimization is that it does not alter any of the amino acids encoded by the *FVIII* or *FIX* transgene obviating possible immunogenicity concerns. Typically, codon-usage optimization resulted in a 3-fold increase in *FIX* protein expression levels, but in the case of *FVIII* an unexpectedly greater impact on *FVIII* protein levels was apparent (up to 44-fold).¹⁸ Deletion of the B-domain (*FVIIIΔB*) was also shown to be essential in boosting *FVIII* expression levels, by increasing the *FVIII* mRNA levels compared with the full-length *FVIII* cDNA.²⁰ Nevertheless, deletion of the entire B-domain compromises specific post-translational intracellular *FVIII* trafficking due to the loss of critical glycosylated residues.²¹ To compensate for this defect, a synthetic B-domain-derived 17-amino acid polypeptide was reintroduced in the *FVIIIΔB* cDNA that presumably reconstitutes these asparagine-linked oligosaccharides, consistent with a ~3-fold increase in the secreted *FVIII* protein levels.¹⁹ Alternatively, incorporation of a furin cleavage site within the B domain (position R1645H) that mimics the canine sequence (histidine-histidine-glutamine-arginine [HHQR] vs. human arginine-histidine-glutamine-arginine [RHQR]) increased *FVIII* expression ~2-fold in hemophilia A mice.²² *FVIII* expression levels could also be increased by generating porcine-human hybrid *FVIIIΔB* transgenes.²³ It has been shown that overexpression of *FVIII* above 200% may provoke cellular stress which may in turn increase the risk of inhibitors.²⁴ It may therefore be prudent to restrict *FVIII* expression levels to minimize the risk of such untoward cellular stress responses. However, it is

reassuring that *FVIII* levels up to 75-fold higher than physiologic levels did not result in detectable transaminitis or liver toxicity.²⁵

As an alternative to codon optimization, we showed, for the first time, with liver-directed gene delivery that a relatively robust increase in *FIX* activity (by up to 8-fold) could be obtained by incorporating a single R338L gain-of-function point mutation in the *FIX* gene.^{13,26} This point mutation (designated as *FIX*-Padua) was initially discovered in thrombophilic patients²⁷ and this could be harnessed to significantly augment the efficacy of gene therapy for hemophilia B with minimal sequence perturbation. The functional enhancement of this specific point mutation was confirmed in subsequent studies.^{28–30} Several other mutants have been explored that enhance *FIX* activity.³¹

Target cells for hemophilia gene therapy

Different cell types have been explored for gene therapy of hemophilia and have been extensively reviewed elsewhere.^{4,5} Some of the most relevant cell types are discussed in more detail below.

The liver is an attractive target organ for both hemophilia A and B gene therapy since *FVIII* and *FIX* are naturally produced by liver sinusoidal endothelial cells (LSECs) and hepatocytes, respectively. Consequently, LSECs and hepatocytes have been targeted by different viral and nonviral vector³² systems for gene therapy. Perhaps most importantly, the hepatic niche may favor the induction of immune tolerance towards the transgene product,³³ which may depend, at least in part, on the induction of regulatory T-cells.^{34,35}

Skeletal muscle cells have a relatively robust secretory capacity and are equipped with the necessary cellular machinery enabling the post-translational modifications required to generate functional *FIX*. Though *FIX* could be secreted into the circulation when expressed in the muscle, off-loading *FVIII* in the circulation is impaired compared with the liver. This may be due to the relatively large size of *FVIII* particularly when complexed with vWF. Unlike liver-directed gene transfer, it would appear that muscle-directed approaches appear to have a higher risk of inducing immune response against the transgene product.³⁶ Despite these limitations, early clinical trials showed that muscle can give rise to sustained *FIX* expression in patients up to 10 years after gene therapy.³⁷

Hematopoietic stem cells (HSCs) have also been explored as targets for hemophilia gene therapy to specifically express *FVIII* or *FIX* in different hematopoietic lineages. In particular, HSC-derived erythrocytes, megakaryocytes and their platelet

progeny could serve as a delivery platform to secrete the clotting factors directly in the circulation following HSC-targeted gene transfer.³⁸

Preclinical and clinical studies

Gene therapy for hemophilia A and B requires an efficient and innocuous gene transfer system that should preferably give rise to sustained, life-long therapeutic FVIII or FIX expression without any immune or toxic side effects. Several vectors have been developed that are well suited for gene therapy of hemophilia, each with their own advantages and limitations.

Nonviral vectors

Nonviral vectors typically rely on DNA-based physical or chemical transfection methods. Though these methods typically result in no adaptive immune responses, as opposed to when viral vectors are employed, it is well established that DNA can evoke innate immune responses *in vivo*, typically involving Toll-like receptor signaling pathways. The gene transfer efficiencies are generally lower compared with most viral vector-mediated gene transfer approaches. Moreover, nonviral transfection of *FVIII* or *FIX*-containing constructs typically results in short-term expression of the transgene product, unless selection is applied on *ex vivo* transfected cells.³⁹ A phase 1 clinical trial for hemophilia A had previously been conducted with stably transfected autologous fibroblasts that were electroporated with *FVIII*-expressing plasmids. After selective expansion, the transfected fibroblasts were implanted into the patient's omentum. Though no adverse events were noted, the therapeutic efficacy was modest and no sustained *FVIII* expression could be attained.⁴⁰ We and others have shown that the efficiency of stable genomic integration and *FIX* or *FVIII* expression levels could be substantially increased using transposons or integrases, consistent with sustained phenotypic correction of the bleeding phenotype in hemophilic mice.^{15,41–43}

Gamma-retroviral and lentiviral vectors

Gamma-retroviral vectors integrate stably into the chromosomes of actively dividing cells. In 1999, we had established the first proof-of-concept that hemophilia A could be cured by gene therapy in a preclinical model of hemophilia A.⁴⁴ To achieve this, neonatal *FVIII*-deficient mice were used since they are permissive for γ -retroviral transduction by virtue of the rapid hepatocyte turn-over. This was subsequently confirmed in neonatal hemophilia A and B dogs.^{45,46} A phase 1 clinical trial was conducted in adult hemophilia A patients but *FVIII*

levels were low due to the requirement for cell division.^{47,48} In contrast, lentiviral vectors could transduce quiescent noncycling hepatocytes, leading to relatively efficient transduction in adult livers (reviewed in Matrai *et al.*^{49,50}). We demonstrated that lentiviral vectors could also transduce professional antigen-presenting cells (APCs) (*i.e.*, Kupffer cells) and LSECs.⁵¹ Inadvertent expression of *FVIII* or *FIX* in APCs increases the risk of developing inhibitory antibodies that preclude their long-term expression and renders gene therapy ineffective.^{52,53} Recent studies revealed that residual *FIX* expression in CD11b⁺ plasmacytoid dendritic cells contributes to this immune response, whereas *FIX* expression in conventional dendritic cells or LSECs contributes to immune tolerance instead.⁵⁴ The mechanism of immune tolerance induction following hepatic *FVIII* or *FIX* delivery with lentiviral vectors requires induction of regulatory T cells.^{17,34} Long-term *FIX* expression could be achieved by preventing expression in APCs through the use of hepatocyte-specific promoters.^{52,53} In some cases, fine-tuning the specificity of expression using microRNA-regulated expression cassettes was required to achieve long-term expression and immune tolerance.^{16,26,54,55} We have shown that *FIX* activity could be further increased by using a codon-optimized hyperactive *FIX*-R338L (Padua) after transduction in hemophilic mice with integration-competent or integration-defective lentiviral vectors.²⁶ Moreover, sustained therapeutic *FIX* expression levels could be attained after liver-directed lentiviral transduction in hemophilic dogs,⁵⁶ paving the way toward eventual clinical translation. One advantage of lentiviral vectors is the absence of pre-existing, vector-specific immunity since most individuals have not been naturally pre-exposed to the lentiviral vector components, in contrast to adeno-associated viral (AAV) vectors (see below). Nevertheless, by virtue of their ability to efficiently interact with professional APCs, lentiviral vectors are capable of inducing innate immune responses, consistent with increased production of pro-inflammatory cytokines.⁵³

Gamma-retroviral and lentiviral vectors have been employed to deliver *FVIII* or *FIX* genes to HSCs and other stem/progenitor cells populations (reviewed in previous publications by Matri *et al.*^{49,50} and Vandendriessche *et al.*⁵⁷). Transplantation of HSC transduced with *FVIII* or *FIX* γ -retroviral or lentiviral in myeloablated recipient mice resulted in phenotypic correction in hemophilic mice and induction of immunological tolerance to the transgene products. One of the attractive features of lentiviral transduction of HSCs is that

FVIII and FIX expression can be specifically directed to platelets resulting in hemostatic correction in both mouse and dog models of hemophilia.^{38,58,59}

As activated blood platelets mediate the primary response to vascular injury, storage of FVIII within platelets may provide a locally inducible treatment to maintain hemostasis for hemophilia A. Remarkably, phenotypic correction of the bleeding disease in hemophilia A mice could even be achieved in the presence of high-titer inhibitory antibodies after lentiviral platelet-directed FVIII expression, which requires von Willebrand factor.^{57,60,61} This strategy could be especially beneficial to treat hemophilia patients with pre-existing inhibitors.

To facilitate HSC engraftment and create a “niche” in the bone marrow, it is necessary to use busulfan or other cytotoxic preconditioning regimens. However, since preconditioning is not without side effects, the risk/benefit ratio would need to be carefully assessed in the context of HSC-based hemophilia gene therapy. To obviate the need for myeloablative preconditioning, direct intraosseous delivery of lentiviral-*FVIII* vectors has been explored. This resulted in bone marrow and HSC cell transduction *in situ* and subsequent FVIII production in HSC-derived platelets.⁶² Alternatively, B cells could be employed, since they yielded therapeutic FIX levels *in vivo* in xeno-transplanted immunodeficient mice without preconditioning.⁶³

One of the main safety concerns related to γ -retroviral and lentiviral vectors relates to the risk of insertional oncogenesis resulting from random genomic integration.⁵⁰ The vector design or the presence of transcriptionally active long terminal repeat can influence this genotoxic risk. Removal of approximately 400 bp in the long terminal repeat region to abolish its transcriptional activity (*i.e.*, self-inactivating vector design) coupled with the use of a promoter in an internal position substantially lowers the risk of insertional oncogenesis. The relative safety of these self-inactivating vectors has been confirmed experimentally in sensitive tumor-prone mouse models using lentivirally transduced HSCs or hepatocytes.^{56,64} This implies that the vector design itself can dramatically decrease the risk of insertional oncogenesis even in tumor-prone mouse models that overestimate this risk. Integration-defective lentiviral vectors that harbor an inactivating mutation in the integrase further minimize the risk of insertional oncogenesis. We showed that transgene expression levels were reduced in comparison with conventional integrase-competent lentiviral vectors.⁵⁵ Nevertheless, relatively robust FIX activity levels could be obtained with integration-defective lentiviral

vectors when the FIX-R338L Padua variant was employed.²⁶

Adenoviral vectors

Adenoviral vectors have been extensively studied for hemophilia gene therapy but were largely abandoned after the failed adenoviral vector trial to treat ornithine transcarbamylase deficiency in the late 1990s. Their main disadvantage pertains to the risk of uncontrollable inflammatory reactions following systemic administration that can potentially be fatal. Nevertheless, we and others have shown that high-capacity adenoviral vectors devoid of any viral genes and encoding FVIII or FIX gave rise to robust supraphysiologic clotting factor expression levels, with limited toxicity and no evidence of inflammation in hemophilic mouse and dog models.^{25,65,66} A phase 1 clinical trial was conducted in severe hemophilia A patients, based on the systemic administration of a high-capacity adenoviral vector encoding FVIII. Although very low FVIII levels (approximately 1%) may have been obtained, the trial was stopped when a transient inflammatory response with hematologic and liver abnormalities became apparent after the first patient was treated. Though the latest-version high-capacity adenoviral vectors result in reduced adaptive immune responses and improved stability of transgene expression, the innate immune response in human subjects is difficult to predict and can still be a serious issue. Consequently, it may not be straightforward to simulate the adenoviral vector-induced innate immune response in pre-clinical animal models. Current efforts are aimed at minimizing the interaction between high-capacity adenoviral vectors with the innate immune system (*e.g.*, by localized delivery⁶⁷) before contemplating future clinical trials with this type of vector for hemophilia gene therapy. However, this will be a tough call given the promising developments with other safer vector systems, particularly AAVs.

Adeno-associated viral vectors

Hemophilia B. AAV2 vectors can transduce a broad variety of tissues, including muscle and liver. Muscle-directed gene transfer in a mouse or canine hemophilia B model with a missense mutation resulted in stable FIX expression.^{68,69} However, in dogs with a *FIX* null mutation, high-titer inhibitors could be induced curtailing phenotypic correction.⁷⁰ The overall therapeutic efficiency was increased up to 10-fold by delivering the AAV-*FIX* vectors to the muscle intravascularly, under transient immunosuppression.⁷¹ The risk of inhibitor

formation was determined by the underlying mutation in the *FIX* gene, vector dose, and local *FIX* antigen doses in the transduced muscle and could be prevented by transient immune suppression or by limiting the vector dose per site (reviewed in Wang *et al.*⁷²). Based on these preclinical studies, a phase 1 clinical trial was initiated whereby severe hemophilia B patients with a missense *FIX* mutation were injected at multiple intramuscular sites with AAV-*FIX* vectors.⁷³ Though circulating *FIX* levels were low or subtherapeutic, *FIX* expression was detectable locally in the muscle for at least 10 years with no evidence of anti-*FIX* antibodies.³⁷

The need for repeated muscle injections, and the increased inhibitor risk, justified exploring AAV-mediated hepatic gene transfer as an alternative. Initially AAV2 was used, followed by other naturally occurring serotypes (*i.e.*, AAV8, AAV9, AAV5) or capsid variants obtained by evolution and selection^{74,75} or containing specific point mutations.⁷⁶ Preclinical studies with the AAV vectors in murine and canine models of hemophilia or in nonhuman primates have demonstrated persistent therapeutic *FIX* expression, leading to partial or complete correction of the bleeding phenotype.^{10,12,53,77–82} Long-term correction of the bleeding disease could be achieved after liver-directed AAV2-*FIX* gene therapy even in inhibitor-prone hemophilia B dogs harboring a *FIX* null mutation.⁸⁰ It would appear, therefore, that the risk of inhibitor development is reduced after liver-directed gene therapy compared with muscle-targeted therapy. This may possibly be due to the induction of regulatory T cells.^{33,83}

These liver-directed preclinical gene therapy studies justified the use of AAV vectors in a phase 1 clinical trial in patients suffering from severe hemophilia B. It was particularly encouraging that therapeutic *FIX* levels, reaching nearly 12% of normal levels, could be achieved following liver-directed delivery of AAV-*FIX*.⁸⁴ However, *FIX* expression was transient and resulted in vector dose-dependent hepatotoxicity consistent with plasma elevation of transaminases. One possible hypothesis (designated hereafter as the “T-cell hypothesis”) that could account for this observation is that the transduced hepatocytes were able to present AAV capsid-derived antigens in association with major histocompatibility complex class 1 to T cells, which would explain the enzymed-linked immunoSpot (ELISPOT) results from patient peripheral mononuclear blood cells (PMBCs).^{84,85} Although antigen presentation was modest, it may have been sufficient to flag the transduced hepatocytes for T-cell-mediated destruction. Though attempts were made to simulate this AAV-specific T-cell response in

various model systems *in vitro* and *in vivo*,^{86,87} it remains somewhat puzzling why this has not been observed in more conventional preclinical disease models (mouse, dogs) or in nonhuman primates.

In a second liver-directed gene therapy trial, severe hemophilia B patients were injected intravenously with an AAV8 vector expressing a codon-optimized *FIX* from a liver-specific promoter.^{57,88} This trial was based, at least in part, on the rationale that AAV8 allows for a substantial increase in hepatic transduction compared with other serotypes,^{89,90} though this may not necessarily translate to higher species, including humans. This AAV8 serotype exhibits reduced cross-reactivity with pre-existing anti-AAV2 antibodies. Interestingly, its uptake by dendritic cells may be reduced compared with conventional AAV2 vectors, potentially resulting in reduced T-cell activation based on preclinical studies.⁹¹ The AAV vector used in this trial was based on a so-called self-complementary design,⁸² which was believed to overcome one of the limiting steps in AAV transduction, namely the single-stranded DNA to double-stranded transcription-competent double-stranded DNA conversion.⁹² Nevertheless, its effect on expression may vary depending on the expression cassette. The treated subjects expressed *FIX* above the therapeutic 1% threshold for several years after vector administration, yielding sustained expression levels ranging between 1% and 6% of normal levels over a median period of 3.2 years. This demonstrated, for the first time, that sustained therapeutic *FIX* levels could be achieved after gene therapy in hemophilia B patients. In the high-dose group (2×10^{12} vg/kg), a consistent increase in the *FIX* level to $\sim 5\%$ was observed in all 6 patients, which resulted in a reduction of more than 90% in both bleeding episodes and the use of prophylactic *FIX* concentrate. In this trial, AAV-specific T-cell responses could be detected, but not in all of the patients.

A recent trial based on the same vector design (UniQure; NCT02396342) but using another capsid (AAV5) essentially confirmed these results. In those patients ($n = 5$) who received the initial dose level (5×10^{12} vg/kg) of the AMT-060 vector, an average of $\sim 4\%$ *FIX* was attained. In the higher-dose cohort (2×10^{13} vg/kg) ($n = 5$), *FIX* expression increased to almost 7%. This was consistent with a reduced spontaneous bleeding rate and *FIX* usage to the extent that eight out of nine participants who were on *FIX* prophylaxis initially did not require prophylaxis any longer. In this trial, AAV-specific T-cell responses could not be detected.

Based on these trial results no apparent benefit of using AAV5 over AAV8 was apparent in terms of

efficacy. Nevertheless, it is challenging to directly compare these trial results since there were no head-to-head comparisons and there may be many confounding variables that impact on the trial outcome, besides the capsid itself (*i.e.*, ratio of functional to total vector particles, differences in manufacturing and vector purity, patient-dependent characteristics, etc.). Strikingly, in both these AAV8 and AAV5 trials, elevated transaminase levels were apparent, consistent with the result obtained in the initial AAV2 trial.⁸⁴ Patients were therefore treated by transient immune suppression with tapering doses of glucocorticoids in the hope of blocking any inadvertent immune responses. Though there is circumstantial evidence supporting the “T-cell hypothesis,” some patients in the AAV8 trial had no elevated liver enzyme levels despite an increased in AAV capsid-specific T-cell response. Other patients in both the AAV8 or AAV5 trial had an increase in liver enzyme levels, without any supporting evidence of an AAV capsid-specific T-cell response. This suggests detecting vector-specific T-cells in the peripheral blood may not necessarily be associated with the immune rejection of AAV-transduced hepatocytes, perhaps due to the impact of the liver microenvironment on the local T-cell response. Alternatively, the liver enzyme elevation in some of the subjects may have been unrelated to an AAV-specific T-cell response or the T-cell response may not have been sufficiently robust to clear the transduced hepatocytes. The availability of new mouse models that mimic these responses may ultimately shed light on these controversies.⁸⁷

Based on these AAV2, AAV8, and AAV5 trials, there is a need to further improve the overall efficacy to the extent that lower and safer effective vector doses could be used that, ideally, would not result in transaminitis. We had initially demonstrated that liver-directed gene transfer of a codon-usage optimized, hyperfunctional *FIX R338L* Padua variant resulted in a significant dose advantage without any apparent increased thrombotic or immunogenic risk compared with wild-type *FIX*.^{13,26} This was independently confirmed in other studies, including canine models.^{29,30} Consequently, this justified the use of a hyperfunctional *FIX-R338L* Padua variant to treat hemophilia B patients by gene therapy.

It is particularly encouraging that the superiority of the *FIX-R338L* Padua was recently supported by two independent clinical trials. In the first trial (Shire, NCT01687608), patients were treated with a scAAV8 vector encoding a codon-optimized *FIX-R338L* Padua variant (BAX335).

Seven patients were treated in three dosing cohorts, and 2 patients had transient *FIX* activity >50%. *FIX* levels persisted in 1 patient from the medium dose cohort of 1×10^{12} vg/kg. None of the patients developed *FIX* inhibitors. In a second trial (Spark Therapeutics/Pfizer), a more consistent response among the trial subjects was apparent. The single-stranded AAV vector genome in the *SPK-9001* vector (NCT02484092) contained a codon-optimized *FIX-R338L* Padua variant and was packaged using an alternative mutated capsid mutant. The mean steady-state *FIX* activity level for the 10 participants, 12 weeks treated at the initial dose level (5×10^{11} vg/kg), was sustained at 33% with no evidence of inhibitory antibodies. Annualized bleeding rate among the 10 patients was reduced by 96% to a mean of 0.39 annual bleeds, compared with 9.2 bleeds before SPK-9001 administration. In addition, annualized infusion rates were reduced 99% to a mean of 0.98 annual infusions, compared with 68.5 infusions before SPK-9001 administration. *FIX* prophylaxis was discontinued. However, 2 patients who showed a transient asymptomatic elevation in liver enzymes and drop in *FIX* activity, potentially indicating an immune response, were treated with oral corticosteroids.

Hemophilia A. The packaging constraints of AAV initially hampered vector production for hemophilia A gene therapy due to the relatively large size of the *FVIII* transgene. Typically, relatively high vector doses were required and it remained challenging to accommodate the *FVIII* transgene with a potent hepatocyte-specific promoter in a single vector. The use of single-chain was preferred over dual-chain vector expressing the heavy and light chain separately.^{93–96} The efficacy of gene therapy for hemophilia A using AAV vectors could be enhanced by using small regulatory elements to drive expression of a B-domain-deleted form of *FVIII* by using codon-optimized *FVIII* cDNA and/or incorporating specific mutations in the *FVIII* cDNA.^{19,22,96–99} Hemophilia A dogs that received AAV2-*cFVIII*, AAV6-*cFVIII*, and AAV8-*cFVIII* persistently expressed therapeutic levels of *FVIII*, without antibody formation or other toxicities, for more than 3 years. However, in contrast to mice, liver transduction efficiencies are similar between AAV2, AAV6, and AAV8 serotypes in hemophilia A dogs.

In a more recent study, an AAV8 vector was constructed that contained a 5.2kb vector genome encoding a codon-optimized *FVIIIΔB* cDNA. A 17-amino-acid peptide (V3) containing glycosylation residues was incorporated in the *FVIIIΔB*

sequence that typically boosted FVIII expression ~3-fold. Supraphysiologic hFVIII expression levels ($732 \pm 162\%$ of normal) could be attained in hemophilia A mice that were injected with 2×10^{12} vg/kg. Stable hFVIII expression at $15 \pm 4\%$ of normal was observed at this dose in a nonhuman primate receiving the lowest vector dose (2×10^{12} vg/kg). However, human FVIII expression above 100% was observed in three macaques that received a higher dose resulting in neutralizing anti-FVIII antibodies that were abrogated with transient immunosuppression. Whether this V3 element will increase the risk of inhibitors in patients with hemophilia A remains to be seen.

A phase 1/2 clinical trial was recently initiated (BioMarin, NCT02576795) based on an AAV5 vector (designated as BMN270) that expressed a B-domain deleted *FVIII* (identical to Refacto^{TM}). A total of 9 patients have received a single dose of the BMN270 vector, and 7 of those have been treated at the highest dose. Out of 9 patients, 6 patients who received the highest dose of 6×10^{13} vg/kg attained FVIII levels above 50%, and one had FVIII levels above 10%, after a follow-up of 16 weeks. The therapeutic outcomes improved over time, with 4 patients reaching 146% FVIII level by week 20. For the 6 patients at the high dose and previously on a FVIII prophylactic treatment regimen, the mean annualized bleeding rate dropped 91% from 16.3 before receiving BMN 270 to 1.5 two weeks after receiving the therapy. For those same 6 patients, the mean annualized FVIII infusions fell 98% from 136.7 to 2.9. Nevertheless, those patients receiving high doses of vectors required a steroid regimen for up to 2 weeks after the gene therapy, whereas the maximum transaminase levels were about twice the upper limit of normal at about 12 weeks after the gene delivery, which then declined over the next few weeks. The vector doses used in this trial were substantially higher than what had been used in the AAV-based hemophilia trials. This poses significant vector manufacturing challenges and may have accounted for the unusual transaminase kinetics.

CONCLUSIONS AND PERSPECTIVES

Is gene therapy for hemophilia now ready for prime time? This is the “million-dollar question”—literally and figuratively, given the high cost of current treatment (PST costs an estimated average \$300,000/year per patient). The prospect for a real cure for hemophilia patients has never looked more promising than it does today. This was made possible through a concerted effort of many investi-

gators that contributed to improved vector development and design, vector manufacturing, basic studies in coagulation, immunology and vector–host interactions at the molecular and cellular level, and proof-of-concept studies in preclinical disease models. This required an unprecedented multidisciplinary effort involving basic scientists, clinicians, regulators, and stakeholders from industry that fueled the gene therapy pipeline. The advances in the clinic progressed through several logical stages starting with the demonstration that transient therapeutic clotting factor levels could be attained, to more sustained and higher FVIII and FIX expression levels and activities, reducing bleeding frequency and clotting factor usage, even obviating the need for prophylaxis. In particular, the use of bioengineered clotting factors (*i.e.*, based on codon-optimized *FVIII* or *FIX* and/or hyperfunctional *FIX* variants) and improved vector designs was important to bring gene therapy for hemophilia to the next level. Nevertheless, there are still some issues that will need to be addressed. Traumatic bleeds still occur in patients, and the issue of vector-induced liver inflammation will need to be better understood and overcome. Moreover, the potential risk of insertional oncogenesis continues to raise controversy.^{100–107} Nevertheless, the emergence of efficient site-specific integrating vectors based on designer zinc finger nucleases, CRISPR/Cas9, or nuclease-free targeting approaches into “safe harbor” loci opens new perspectives to further minimize this risk.^{108–111,112} The careful selection of potent *cis*-regulatory elements that boost *FIX* or *FVIII* expression without any apparent increased risk of insertional oncogenesis is warranted.¹⁵

An adequate balance between efficacy and safety will need to be continuously assessed in the face of new insights in this rapidly developing field, particularly if the number of patients are expected to substantially increase in the coming years as gene therapy trials move into phases 3 and 4. Eventual marketing authorization approval by regulatory authorities depends on the successful completion of these larger trials. Only then will gene therapy for hemophilia make a concrete difference in the lives of patients and their families that are affected by these diseases.

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AUTHOR DISCLOSURE

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