

# Developments in the treatment of hemophilia B: focus on emerging gene therapy

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**Abstract:** Hemophilia B is a genetic disorder that is characterized by a deficiency of clotting factor IX (FIX) and excessive bleeding. Advanced understanding of the pathophysiology of the disease has led to the development of improved treatment strategies that aim to minimize the acute and long-term complications of the disease. Patients with hemophilia B are ideal candidates for gene therapy, mostly because a small increase in protein production can lead to significantly decreased bleeding diathesis. Although human clotting FIX was cloned and sequenced over 30 years ago, progress toward achieving real success in human clinical trials has been slow, with long-term, therapeutically relevant gene expression only achieved in one trial published in 2011. The history of this extensive research effort has revealed the importance of the interactions between gene therapy vectors and multiple arms of the host immune system at multiple stages of the transduction process. Different viral vector systems each have unique properties that influence their ability to deliver genes to different tissues, and the data generated in several clinical trials testing different vectors for hemophilia have guided our understanding toward development of optimal configurations for treating hemophilia B. The recent clinical success implementing a novel adeno-associated virus vector demonstrated sufficient FIX expression in patients to convert a severe hemophilia phenotype to mild, an achievement which has the potential to profoundly alter the impact of this disease on human society. Continued research should lead to vector designs that result in higher FIX activity at lower vector doses and with reduced host immune responses to the vector and the transgene product.

**Keywords:** hemophilia B, factor IX deficiency, adeno-associated virus, adenovirus gene therapy

## Overview of pathophysiology and genetics of hemophilia

Hemophilia is a genetic disorder characterized by the deficiency of a plasma protein needed for normal blood clotting. The two most common forms of hemophilia are hemophilia A and B; both are classically transmitted in an X-linked recessive pattern, with one third of cases due to de novo somatic mutations.<sup>1</sup> Hemophilia occurs in one in 5,000 live male births, and of these, 80% are hemophilia A and 20% are hemophilia B. In patients with hemophilia B, the mutation occurs in Xq27.1 and leads to an absence of functional blood coagulation Factor IX (FIX). The incidence is one in 25,000 males. Females with a mutated FIX gene are typically carriers, but can occasionally have exceptionally low factor levels, apparently by non-random X chromosome inactivation.<sup>2</sup>

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FIX plays a crucial role in the coagulation pathway. The tissue factor pathway for Factor X activation requires Factor VIII and FIX for normal thrombin generation; the absence of either protein severely impairs the ability to generate thrombin and fibrin. Thrombin is necessary for platelet aggregation, fibrin generation, clot retraction, and activation of Factor XIII. Because thrombin generation in hemophilia is markedly delayed, hemorrhage may occur after minimal or unknown trauma. Moreover, the clot formed is often friable, and rebleeding is a common observation in inadequately treated patients. Deep bleeding into joints and muscles is characteristic of hemophilia.

The bleeding phenotype of hemophilia B is classified as mild, moderate, or severe, based on widely accepted clinical assays for FIX activity. One International Unit of FIX activity is defined as the amount of FIX activity in 1 mL of normal plasma; therefore, people with normal FIX levels average 100 IU/dL or 100%. According to the Scientific and Standardization Committee of the International Society on Thrombosis and Hemostasis, patients with mild hemophilia have FIX levels of 5–40 IU/dL, those with moderate disease have 1–5 IU/dL, and patients with severe disease have <1 IU/dL. Patients with mild hemophilia may often go undiagnosed until challenged with surgery or significant trauma, whereas patients with moderate hemophilia experience bleeding after mild to moderate injury. In contrast, patients with severe disease present in infancy and often have bleeding episodes after minimal trauma or may have spontaneous hemorrhage despite the use of prophylactic FIX concentrate. Molecular genetic testing of FIX identifies disease-causing mutations in more than 99% of individuals with hemophilia B. Over 50 years of research into this disease has advanced our understanding of the pathophysiology of the disease and led to the development of powerful tools for diagnosis and improved treatments which minimize the acute and long-term complications of the disease and its therapy.

Molecular characterization of this disease began in 1952, when FIX was discovered to be lacking in a child with hemophilia B named Stephen Christmas. The original disease mutation was identified by sequencing the child's DNA, revealing a mutation which changed a cysteine to a serine at position 206 in the FIX protein. In the 1950s and 1960s, the standard of care for hemophilia consisted of whole blood or fresh plasma transfusions. Unfortunately for patients with severe disease, the quantity of clotting factors in these transfusions was not sufficient to correct serious bleeding, so many died in childhood. By the mid 1960s, cryoprecipitate and plasma purified clotting factors were identified

as being therapeutic for patients with hemophilia. In the 1970s, plasma-derived FVIII and FIX concentrates became available for use at home. This development considerably reduced hospital visits and greatly improved quality of life for patients with hemophilia.

A decade later, however, with the emergence of human immunodeficiency virus (HIV)/acquired immune deficiency syndrome, approximately 50% of patients with hemophilia went on to become infected with the virus via blood transfusions. Because of this, the efforts to advance the knowledge of hemophilia and to develop safer treatments continued. The gene for FIX was first cloned by Kurachi and Davie<sup>3</sup> and for Factor VIII by Gitschier et al,<sup>4</sup> which soon after led to approval of the first recombinant FIX and FVIII concentrates by the US Food and Drug Administration. The availability of these synthetic factors made treatment of hemophilia safer and led to another important trend in treatment for the disease, ie, the use of scheduled factor infusions (prophylaxis) to prevent chronic bleeding episodes. Although these important advances have greatly reduced the morbidity and mortality associated with hemophilia B, there are still significant problems with existing therapy, notably that robust prophylactic coverage in severe patients requires multiple infusions per week. Repeated bleeding episodes still occur in many patients for whom prophylactic therapy is available, and can cause long-term damage in joints and other tissues.

## Gene therapy: a new paradigm

Gene therapy can theoretically eliminate these issues by stably inserting the gene for FIX into a patient's tissue which then permanently restores clotting factor levels in the circulation for the life of the patient. Soon after the gene was cloned, retroviral vectors encoding the human FIX complementary DNA were used to infect skin fibroblasts, which then could produce circulating FIX at readily detectable levels in rats and mice.<sup>5</sup> Unfortunately, it has been enormously difficult to translate this early success in laboratory animals into the clinic, and therapeutically significant and stable FIX expression was only just demonstrated in hemophilia B patients for the first time in 2011,<sup>6</sup> after more than two decades of intense research effort.

Before describing these recent clinical results, which utilized the adeno-associated virus (AAV) vector system to transduce the liver, and how they were made possible, it is helpful to first summarize a few critical concepts of gene delivery technology. Introduction of the gene into a patient's cell is called transduction, and the vehicle used is known as the vector. The target cell can either be in culture, which

allows ex vivo transfer, or reside in an organ, which requires in vivo delivery. There are broadly two categories of vectors, ie, nonviral vectors (naked DNA, DNA encapsulated with liposomes) and viral vectors. Nonviral vectors can have reduced toxicity and are more easily produced, but are not very efficient for transducing primary cells. Viruses naturally have the inherent ability to gain access to specific cells and transfer the genetic material they carry. Recombinant vectors are derived from wild-type viruses and are typically modified so that they retain the ability to enter cells but cannot replicate in the transduced cell. This is accomplished by deleting or mutating sequences from the vector that code for viral replication proteins, and so these proteins must be expressed in the cells that are used to manufacture the viral vector product. In place of the replication protein genes, vectors contain expression cassettes that encode the gene of interest, which in the case of hemophilia B is FIX.

Viral vectors can be nonintegrating or integrating, depending on the final status of the delivered genetic material. Nonintegrating viral genomes are maintained as episomes, and include herpes virus, adenovirus, and AAV vectors. Transgene expression from nonintegrating vectors may be transient because episomes are lost with each cell division and the rate of decline in expression is dependent upon the rate of cell division and turnover of the transduced cells. Retroviral vectors, on the other hand, covalently integrate the genetic cargo into the host cell chromosome, thus enabling the delivered gene to be copied along with the host genetic material each time the cell divides. Retroviral gene therapies increasingly utilize vectors based on HIV, which is in the lentivirus family and is uniquely efficient at transducing nondividing and terminally differentiated cells.<sup>7,8</sup> HIV-based lentiviral vectors have been used to directly transduce the liver for the treatment of hemophilia in mouse models,<sup>9,10</sup> but the levels of circulating factor expression achieved are still low relative to episomal vector systems, and have not progressed beyond animal models.

The nonintegrating vectors most frequently utilized for hemophilia gene therapy are adenovirus and AAV. The results obtained in clinical trials using adenovirus have significantly influenced the subsequent development of AAV vectors, and so although AAV is becoming the vector of choice for hemophilia B, it is helpful to review the properties of both vector systems. Adenovirus is a common respiratory virus with a nonenveloped icosahedral particle ~90 nm in diameter and an approximately 36 kb double-stranded linear DNA genome, which in the first generation vector (FG-Ad) were deleted in the E1 gene region (or combinations of E1 and E3 or E4) to

allow for insertion of transgene expression cassettes. Later generation vectors (termed helper-dependent adenovirus, or HD-Ad), replaced all of the adenoviral coding sequences to allow larger transgene capacity and reduced cytotoxicity from residual viral gene expression. Adeno-associated virus was discovered in 1965 as a contaminant of adenovirus preparations. Although like adenovirus, it is nonenveloped and icosahedral, it is smaller than adenovirus (25 nm), and packages a linear single-stranded DNA genome ~4.7 kb in length and of both positive and negative polarity. It belongs to the family Parvoviridae and genus Dependovirus, because robust replication of AAV occurs only in the presence of a helper virus, either adenovirus or herpesvirus.

In addition to the choice of vector system delivering the gene of interest, the tissue or organ targeted is a critical aspect of any gene delivery strategy, and often affects the choice of vector system used. Hemophilia is representative of a large number of diseases that could benefit from systemic circulation of a recombinant protein, and so many investigators have attempted to modify a range of tissues to serve as “depots” for expression of proteins intended to ameliorate the disease state. The choice of target tissue affects the type of vector system that would be expected to be efficacious and profoundly affects the potential for host immune responses to negatively impact the therapy. Some experimental therapies deliver genes to cells isolated from patient tissue prior to reimplantation. This ex vivo approach typically relies on permanent modification of the cell genome by an integrating vector such as a retrovirus or plasmid DNA (that only rarely integrates but can be enriched by selecting cells with drugs). Although early attempts at this transfection and selection approach only provided transient benefit when tested in hemophilia (see below), more recent efforts using lentivirally transduced hematopoietic stem and progenitor cell transplantation are showing significant success in clinical trials for other diseases.<sup>11,12</sup> Although this method of therapy has as of yet not been tested for hemophilia in humans, the ability to treat patients with myeloablative and/or immunoablative conditioning prior to hematopoietic stem and progenitor cell transplantation has the potential to reduce or eliminate the immunological complications that have been observed with other target tissues. Multiple groups have proposed the use of lentiviral vector transduced hematopoietic stem and progenitor cells to treat hemophilia and have shown promising results in animal models.<sup>13,14</sup> Further study is required, however, to determine whether the risk of an ablative conditioning regimen will be offset by the benefits of transduction of long-lived stem cells in this particular disease.

Muscle and liver are common targets for injected gene therapy vectors in immunocompetent individuals, and for these approaches a major factor in the efficacy and safety is the extent to which the host immune system responds to the vector and the transduced cells. This interaction can occur on many levels, here summarized chronologically. First, immediately upon administration of the vector particles, pre-existing humoral immunity can lead to antibody binding and elimination of the vector particles, preventing them from ever reaching the target tissue. As such, strategies attempting to transduce tissue via injection of vector particles into the systemic circulation are more susceptible to this problem than those utilizing direct application of the vector on or in the tissue. Several animal studies have confirmed that such pre-existing antibodies attenuate or abrogate transduction of the liver by AAV vectors,<sup>15,16</sup> while for adenovirus vectors gene transfer can still occur in the presence of pre-existing immunity, but gene expression is somehow attenuated<sup>17,18</sup> and toxic side effects are increased.<sup>19</sup>

A second immunological response can occur via direct stimulation of innate immunity pathways by vector particles. These pathways rely on pattern recognition receptors that recognize a variety of epitopes on pathogenic microorganisms, and lead to cytokine secretion and enhanced stimulation of adaptive immunological responses as well as promotion of direct cytotoxic effects. Early efforts to utilize adenovirus to deliver genes to the liver showed that such innate immune stimulation was critical for an enhanced cytotoxic response to adenovirus vectors, and was instrumental in the tragic death of Jesse Gelsinger, who died of a systemic inflammatory response just 4 days after receiving  $6 \times 10^{11}$  viral particles/kg of an FG-Ad designed to treat his ornithine transcarbamylase deficiency.<sup>20</sup> Another patient treated previously at the same dose with the same vector had only mild, transient side effects,<sup>21</sup> indicating that patient-to-patient variations in the intensity of innate immune responses are significant and perhaps affected by pre-existing adaptive immunity. Subsequent animal studies, however, have failed to replicate the particularly lethal combination of symptoms observed in the ornithine transcarbamylase trial, even with pre-immunization, and so the precise cause of Mr Gelsinger's death remains a mystery.<sup>22</sup>

AAV vectors have been shown to be notably less potent at stimulating innate immune pathways,<sup>23</sup> which appears to be a significant factor in their enhanced longevity of expression in animal models. AAV vectors do, however, weakly stimulate innate pathways, dependent upon the particular vector composition (reviewed by Rogers et al<sup>24</sup>).

Self-complementary AAV vectors (which more rapidly form double-stranded DNA than conventional single-stranded vectors) have been shown to stimulate Toll like receptor 9, which is a known pattern recognition receptor recognizing unmethylated CpG dinucleotides, and this stimulation is stronger than that which occurs with single-stranded AAV vectors.<sup>25</sup> The impact of this pattern recognition receptor stimulation on vector efficacy and design strategies is an active area of investigation.<sup>26,27</sup>

Adaptive immune pathways can also inhibit gene therapies after target cells are successfully transduced by both humoral-mediated and cell-mediated mechanisms. One well appreciated risk for hemophilia gene therapy derives from the potential for the newly expressed clotting factor to stimulate the production of antibodies which bind and inhibit FIX protein. There is a long history of inhibitory antibody development after clotting factor protein replacement therapy in hemophiliacs, and this experience has guided the appreciation of this risk as a consequence of gene therapy. Current gene therapy clinical trials frequently exclude those patients who might be predicted to be more likely to form inhibitory antibodies, and although inhibitor formation has been observed in animal gene therapy studies,<sup>28</sup> it has yet to be observed in human trials. Notably, AAV vectors have been shown to have the potential to induce a state of immunological tolerance toward inhibitor formation in mice,<sup>29</sup> promoting the hope that gene therapy might at some point be a preferred option for treatment of hemophilia patients with inhibitors.

Lastly, cell-mediated adaptive immunity can be responsible for the elimination of vector transduced cells. Classical cytotoxic T-cell (CTL) responses are mediated by CD8+ T-cells that recognize foreign antigens derived from proteins translated in a host cell and positioned in the cleft of major histocompatibility complex (MHC) class I molecules on that cell's surface. Exogenous proteins taken up by a host cell (for example, capsid proteins from viruses that infect a cell) can also be "cross-presented" into MHC class I, which also leads to CD8+ T-cell elimination of infected cells, even when the gene for that protein or peptide is not being delivered to the cell. Early experiments using adenoviral vectors demonstrated that during the several weeks following vector administration, CD8+ T-cells typically infiltrated transduced tissues and appeared to eliminate all transgene-expressing cells, both by recognition of transgene encoded peptides and peptides expressed by the backbone of FG-Ads.<sup>30,31</sup> When the target tissue was the liver, this cytotoxicity was accompanied by transient increases in circulating liver enzyme levels, which are released from liver cells when lysed.

AAV vectors have been shown to have distinct advantages with regard to CTL-mediated destruction of transduced cells relative to adenovirus vectors. For one, CTL responses are strongly stimulated by the cytokines secreted as a result of innate immune stimulation, which is stronger for adenovirus than for AAV.<sup>18,23</sup> Also, AAV vectors are completely devoid of viral genes, unlike first generation adenoviruses. As will be described below, however, CTL responses can still occur during AAV gene therapy, and our understanding of exactly how those responses can be controlled or eliminated in future trials is progressing but limited.

## Hemophilia gene therapy: clinical trial experience

Patients with hemophilia B are ideal candidates for gene therapy because the disease phenotype is attributable to the lack of a single protein that circulates in minute amounts in the plasma, the level of clotting FIX synthesized does not need to be tightly controlled, and the response to treatment can be easily monitored. Moreover, the gene for FIX is small and easy to insert into many vectors, appears to be expressed robustly as a cDNA, and there are good animal models available to test treatment strategies. Finally, years of clinical experience have proven that an increase of just 1%–2% in circulating levels of the deficient clotting factor can significantly reduce the bleeding diathesis. Table 1 summarizes clinical trial experience for gene therapy in hemophilia, including

three clinical trials performed with vector systems other than AAV to treat hemophilia A and which have contributed to our understanding of the challenges facing gene therapy for hemophilia in general.

In 2001, Roth et al<sup>32</sup> described the utilization of plasmid DNA transfection into patient-derived fibroblasts in vitro, drug selection for those cells containing integrated vector DNA, and transplantation of  $1-4 \times 10^8$  genetically modified cells into the patient omentum. Although the therapy in this trial was well tolerated without signs of toxicity, and some of the patients demonstrated measurable increases in circulating Factor VIII levels, all patients failed to maintain detectable expression beyond 10 months.<sup>32</sup> In 2003, Powell et al<sup>33</sup> published on intravenous injection of a  $\gamma$ -retroviral vector (based on Moloney murine leukemia virus) at doses ranging from  $\sim 3 \times 10^7$  to  $9 \times 10^8$  transducing units per kilogram. There was some evidence that vector-mediated augmentation of circulating Factor VIII was achieved in some patients, and there were no observable treatment-related toxicities, but vector derived expression was transient with no measurable clinical benefit in the long-term.<sup>33</sup> In another trial, an HD-Ad was used to treat a single patient at a dose of  $4.3 \times 10^{11}$  viral particles per kilogram, which caused rapid grade 3 liver toxicity (including a ten-fold increase in alanine aminotransferase levels), thrombocytopenia, elevated levels of serum interleukin-6, and laboratory evidence of disseminated intravascular coagulation. All symptoms resolved 19 days post infusion, but the

**Table 1** Clinical trials for gene therapy of hemophilia

Reference	Year	Vector	Gene delivered	Subjects (n)	Administration	Outcome/comments
Roth et al <sup>32</sup>	2001	Plasmid DNA	hFVIII	6	Laparoscopic injection of ex vivo genetically altered fibroblasts into the omentum	Safe and well tolerated. No benefit long-term
Powell et al <sup>33</sup>	2003	$\gamma$ -retroviral	hFVIII	13	Intravenous infusion	Safe and well tolerated. Short-lived circulating FVIII
White and Monahan <sup>34</sup>	2005	HD-Ad vector	hFVIII	1	Intravenous infusion	Severe hepatotoxicity and DIC. No detectable FVIII expression
Manno et al <sup>37</sup>	2003	AAV2	hFIX	8	Intramuscular injection	Safe and well tolerated. First parenteral administration of rAAV in humans. Circulating FIX levels < 1.5%
Manno et al <sup>39</sup>	2006	AAV2	hFIX	7	Hepatic artery infusion	Transient transaminitis. FIX expression detected but only transiently with loss of expression concurrent with transaminitis
Nathwani et al <sup>6</sup>	2011	AAV8	hFIX	6	Intravenous infusion	Mild, transient transaminitis in only some high dose patients. Stable FIX expression > 1% for more than 1 year Effect of transient transaminitis on FIX levels unclear

**Abbreviations:** AAV, adeno-associated virus; DIC, disseminated intravascular coagulation; hFVIII, human coagulation Factor VIII; hFIX, human Factor IX; HD-Ad, helper-dependent adenovirus; rAAV, recombinant adeno-associated virus.

similarity of this toxicity to the symptoms displayed by Jesse Gelsinger suggested that HD-Ads still faced serious toxicity issues in humans.<sup>34</sup> Further clinical development of all of the above therapies has been discontinued.

It was in the context of these observations of vector toxicity and lack of efficacy that researchers began to develop AAV vectors for use in the treatment of hemophilia, predominantly for hemophilia B because the large size of the Factor VIII gene exceeded the capacity for AAV vectors. In 1997, Herzog et al demonstrated that intramuscular injection of a recombinant AAV vector expressing human FIX could direct expression of therapeutic levels of the transgene in experimental mice.<sup>35</sup> In 1999, their laboratory demonstrated sustained expression (>17 months) of FIX in five dogs that received several percutaneous intramuscular injections of AAV at a single time point.<sup>36</sup> Efficient gene transfer to muscle was shown by immunofluorescence staining and DNA analysis of biopsied tissue. Immune responses against FIX were either absent or transient. These data provided strong support for feasibility of the approach for therapy in human subjects.

In 2003, Manno et al published the results of the first AAV clinical gene therapy trial for hemophilia B.<sup>37</sup> The Phase I clinical trial was initiated in 1999 and consisted of an AAV serotype 2 (AAV2) vector encoding FIX being administered via ultrasound-guided intramuscular injection to eight adult patients with hemophilia B. The highest dose administered was  $2 \times 10^{12}$  vector genomes per kg. Muscle biopsies of injection sites performed 2–10 months after vector administration confirmed gene transfer. However, circulating levels of FIX were only 1.0%–1.4% in three cases and <1% in all others, which the authors attributed to their inability to inject either high enough doses of vector or at adequate numbers of sites, based on the risk of inhibitor formation observed in animals. There was evidence that in one patient a level of 1% of normal persisted until 1 year post treatment. The results of this study demonstrated the safety of skeletal muscle AAV administration in humans in a manner similar to that used in mice and hemophiliac dogs. However, the therapy failed to generate adequate levels of circulating FIX, in contrast with the results observed in animal models. Without extensive biopsy analysis of the treated tissue, it is difficult to determine whether the low FIX expression was solely due to inadequate dosing or if other mechanisms contributed, such as gene silencing or CTL-mediated elimination of transduced cells. A 10-year follow-up skeletal muscle biopsy from one of the study participants (who died of unrelated causes) showed persistent FIX expression in injected muscle tissue and AAV vector

sequences, proving that long-term expression of transgenes from muscle was achieved,<sup>38</sup> but was inadequate to achieve significant therapeutic benefit.

Subsequent studies switched target tissue to the liver, the normal site of FIX synthesis. In 2006, Manno et al published the results of a clinical trial where an AAV2 vector with a liver-specific promoter was infused through the hepatic artery in seven subjects.<sup>39</sup> Vector infusion, even at high doses, was not associated with short-term or long-term toxicity. However, an early concern was the appearance of vector DNA sequences in semen, raising the possibility of vertical transmission and potentially altered development of future offspring. The trial was put on hold for several months until animal studies confirmed lack of germline transmission of vector sequences following administration of AAV2 vector,<sup>40</sup> and the protocol was modified to require that subjects practice barrier contraception until two sequential semen samples were negative for vector sequences and consider banking of sperm prior to vector injection.

Other key findings in this trial were transient, apparently CTL-mediated liver toxicity peaking 2–4 weeks after vector infusion, and an inverse correlation between pre-existing neutralizing antibody titers and peak FIX expression.<sup>39</sup> In the two subjects treated with the highest dose of vector ( $2 \times 10^{12}$  vector genomes per kg), the subject with the highest AAV-neutralizing antibody titer (1:17) prior to vector infusion exhibited a peak FIX level of 3% 2 weeks after infusion, although the expression was transient and back to baseline by 4 weeks. The patient who had the lowest antibody titer (1:2) prior to vector infusion showed higher expression of the transgene 2 weeks after vector infusion (12%), but also only maintained FIX expression transiently. This inverse correlation of antibody titer with peak FIX expression supported the hypothesis that neutralizing antibodies can be a critical barrier to gene transfer when delivered via the systemic circulation, as had been observed in animal studies.<sup>15,16</sup> The decline and loss of FIX expression which occurred in both patients (the only patients with detectable FIX expression) was postulated to be occurring via CTL-mediated destruction of transduced hepatocytes, because it was concurrent with a transient asymptomatic elevation of liver transaminases (aspartate aminotransferase and alanine aminotransferase) that peaked as FIX expression declined, much as had occurred in earlier animal and human trials with adenoviral vectors.

These clinical results provided a seminal contribution to the field, and prompted many additional studies investigating

the occurrence of CTLs that target capsid protein-derived epitopes (reviewed by Mingozzi and High<sup>41</sup>). Generation of specific cell staining reagents consisting of capsid epitopes bound to soluble MHC complexes allowed demonstration that human populations rarely contain detectable CTLs that recognize the AAV capsid epitope, but that after stimulation of peripheral blood mononuclear cells with the peptide they are often detectable, and patients treated in the AAV2 clinical trial showed an expansion of this CTL population concurrent with elevation of liver enzymes.<sup>42</sup> The data on the whole support the hypothesis that capsid antigen protein can be cross-presented onto MHC class I complexes and targeted for destruction by reactive CTLs, although it remains to be determined whether other as yet untested epitopes play a role in hepatocyte loss or how the process will be influenced by specific aspects of the vector or the host immune status prior to therapy. It should be mentioned that some have proposed that other antigens could be involved in hepatocyte loss in these patients,<sup>43</sup> or that capsid DNA contaminants in the vector preparation could mediate recognition by capsid-specific CTLs.<sup>44</sup> Although the levels of capsid DNA contamination and expression were convincingly argued to be too low in clinical AAV vector preparations to account for such an effect,<sup>45</sup> the lack of access to actual patient tissue during hepatocyte loss precludes making any definitive conclusions on this important issue.

In 2010, a third AAV-FIX trial in humans was initiated by St Jude Children's Research Hospital and University College London, and capitalized on previous research by introducing several novel features which made a key difference in the overall efficacy. The vector was encapsulated using an alternate AAV serotype (AAV8) which less frequently causes natural infection in humans,<sup>46</sup> less efficiently transduces professional antigen-presenting cells (leading to reduce CTL responses to capsid in animal models),<sup>47</sup> and has increased liver tropism, which enabled peripheral vein infusion of the vector.<sup>48</sup> Patients exhibiting detectable neutralizing antibody titer were excluded. The vector possessed a self-complementary genome for improved transduction efficiency,<sup>49,50</sup> and codon optimization for improved efficiency of transgene expression.<sup>51</sup> Finally, after vector treatment patients were monitored and if found to show evidence of liver inflammation were treated with an immunosuppressive drug. A single infusion of the vector was given to six adult males with severe hemophilia B, with two subjects sequentially assigned to low, intermediate, or high doses of vector. There were no acute toxicities and vector sequences were cleared from excreted fluids within 2 weeks. Notably,

in this trial, all subjects experienced stable FIX expression at 1%–7% of normal levels, when assessed up to 3 years post-treatment.<sup>6</sup>

Although this exciting clinical result has invigorated the field and provided tremendous hope for this therapeutic modality to successfully treat a wide range of diseases, it remains to be determined exactly which specific improvements in the vector and clinical treatment plan were responsible for the improved outcomes relative to previous trials. It is important to consider this question in the context of the fact that the two participants who received the high dose of vector ( $2 \times 10^{12}$  vector genomes per kg) had transient, asymptomatic elevation of serum aminotransferase levels, which although milder in extent and later following vector administration, were similar to what had occurred in the previous liver-directed AAV trial. The overall results are mostly consistent with the predominant theory that capsid protein epitopes are processed from incoming viral particles by hepatocytes and cross-presented on MHC class I, as reviewed by Mingozzi and High.<sup>41</sup> As described in that work, however, this theory still requires significant refinement. Notably, the St Jude/University College London trial used vector which was not density purified, and as such contained in the order of five-fold higher levels of capsid protein than the preparation used in the previous AAV2 trial,<sup>52</sup> even though the targeting of capsid antigens was apparently milder with this product. Additionally, in some subjects treated at lower doses in the St Jude/University College London trial, high levels of CTL reactivity to capsid were observed in peripheral blood mononuclear cells without concurrent elevation of liver enzyme levels. Why were these increases in capsid-targeted CTL reactivity not associated with hepatotoxicity? Continued clinical research will be necessary to explain these conflicting results and more thoroughly define exactly which parameters of the gene therapy and patient physiology affect the hepatotoxicity that has been observed with this exciting new vector.

The overall progress of liver-directed gene transfer for hemophilia and other diseases reveals that the risk of immune-mediated toxicity is multifaceted. Early trials using adenovirus clearly stimulated strong innate and subsequent adaptive immune responses, leading to both toxicity and loss of expression, with large variations in the magnitude of this toxicity between subjects.<sup>20</sup> Although newer AAV-based vectors have significantly reduced this phenomenon, there are still immune-related issues which also show variability between patients. Our understanding of how epitopes are selected for antigen presentation is still primitive,<sup>53</sup> as is

our understanding of tolerance mechanisms in the context of genetic disease. Although careful screening has shown no evidence of CTLs that target the therapeutic FIX transgene, this does not mean that it could not occur in some rare patients, perhaps even prior to therapy, as has been found in patients with Duchenne's muscular dystrophy.<sup>54</sup> Without being confident that CTL activity against FIX-expressing cells could be controlled if it were to occur, continued caution during dosing of hemophilia gene therapy trials is warranted, in particular for patients with residual endogenous expression of the protein. Cytotoxic T-cells have the potential to become dangerous, as demonstrated by the two cell therapy clinical trials in which infused T-cells avoided tolerization mechanisms to profoundly amplify and attack healthy tissue, causing whole organ destruction and death for three research subjects.<sup>55,56</sup>

## Next generation of vectors

Future clinical experiments should address these risks, as well as continue to optimize vectors for improved potency and longevity of expression. A novel mutation in the FIX gene was discovered to enhance its catalytic activity,<sup>57</sup> and this so-called "Padua" mutation has been shown to provide AAV vectors with enhanced potency.<sup>58</sup> This optimization has the potential to enhance functional efficacy in patients by eight-fold, which would significantly reduce the dose required to achieve therapeutic levels of FIX activity. Other alternative serotypes may allow treatment of those patients with pre-existing neutralizing antibodies to AAV serotype 8, and allow retreatment of previously treated patients,<sup>48</sup> but will each require significant investment in preclinical and clinical development. Lastly, continued optimization of the expression cassette design might be predicted to further improve the yield of FIX protein per transduced cell.

A continuing challenge for AAV-mediated gene therapy is that of manufacturing, as the generation of viral vector particles requires a complex cellular process that has been difficult to replicate in robust, scalable stable cell production systems.<sup>59</sup> The product tested in the study by Nathwani et al<sup>6</sup> was made by a relatively cumbersome and labor-intensive transient transfection process,<sup>52</sup> and it remains to be seen whether products derived from other more scalable systems will behave similarly. Relative to other biological drugs, viral vectors are significantly more complex, and it might be expected that different production systems will generate products with subtly different properties that impact clinical efficacy, in particular with regard to stimulation of immune responses.

To date, only ten patients have been treated with the self-complementary vector tested by Nathwani et al,<sup>6</sup> and as such, many large cohorts remain to be tested. Notably, children could receive enormous benefit by the continuous FIX synthesis that gene therapy provides, which would be predicted to significantly reduce the risk of developing long-term joint damage, and children may also have a reduced likelihood of immune complications due to lack of pre-existing immunity to AAV. Pediatric trials must however proceed cautiously, and only after safety and effectiveness has been determined in larger groups of adult patients. One of the challenges for younger subjects is that AAV vectors are predominantly nonintegrating, and when vector is injected into a growing animal, expression is gradually lost from dilution of the episomal vector genomes as cells divide.

The World Federation of Hemophilia estimates that of the 400,000 individuals worldwide with hemophilia, 300,000 receive either no or very sporadic treatment.<sup>60</sup> The high cost of prophylactic regimens hampers widespread use. The median cost of clotting factor products for a patient with hemophilia is in excess of \$50,000 a year.<sup>61</sup> In patients with difficult intravenous access or for children who require frequent injections, insertion of a central venous access device might be necessary for clotting factor administration. This is in turn associated with complications related to central venous line placement and maintenance, including blood stream infections. Despite the use of prophylactic factor concentrates, some patients continue to suffer frequent spontaneous bleeds. The potential for single administration of a gene therapy drug to provide a long-term cure is thus most profound for patients in the developing world, and should be a high priority for all nations to pursue.

Lastly, and perhaps most important, is the development of gene therapy for patients with hemophilia A, which is much more prevalent worldwide. Some of the challenges faced by investigators in this area are difficulty packaging FVIII in recombinant AAV virions due to its large size, low levels of transgene expression, and the greater inherent immunogenicity of FVIII. Despite these limitations, the outlook is promising. McIntosh et al demonstrated therapeutic levels of FVIII in mice and nonhuman primates injected with an AAV vector designed to encode a novel human FVIII variant.<sup>62</sup>

## Conclusion

In the first half of the 20th century, the life expectancy of a patient with hemophilia ranged from 16 to 23 years.<sup>63</sup> In contrast, the life expectancy of patients with hemophilia but not infected with HIV or hepatitis C virus is currently

comparable with that of the general population.<sup>64</sup> The morbidity and cost of care increases proportionally as the life expectancy of patients with hemophilia who have been treated with newer, safer, more efficient factor concentrates increases. FIX levels of just above 1% are associated with a reduction in factor use. This is consistent with the findings of the Swedish prophylaxis studies, which showed a reduction in hemorrhages when concentrate was dosed to maintain nadir levels of approximately 1%.<sup>65,66</sup> However, this high level of care is prohibitively expensive, particularly for populations in the developing world, and there is a strong need for improving treatment options. Gene therapy clearly provides an option with the potential to completely change the course of hemophilia as a disease.

Looking back at the many decades of effort toward hemophilia gene therapy, it is easy to see that although it may have appeared hopeless at times, the previous failed efforts were instrumental in bringing our understanding forward to enable clinical success, as nascent as it is still. Certainly the number one challenge is delivering enough copies of an expression cassette to cells capable of producing the therapeutic protein, but this goal is profoundly influenced by many aspects of the clinical strategy and patient physiology, most critically in the way the immune system responds to the gene delivery event. Importantly, we must realize that we have only just now achieved long-term expression of any protein in significant quantities in the circulation, and so we are only beginning to test the long-term consequences of that event in patients. Most previous gene delivery clinical trials did not have very significant adverse events, but in those trials transduced cells were rapidly eliminated, and so do not represent a real test of the effect of foreign transgenes in human patients in the long-term. Moving forward, we must carefully monitor trial subjects and be careful to continue to balance both the potential risks and the enormous hope of gene therapy.

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## Disclosure

AMD, JTG, and ACN have the potential to share in revenue generated by licensing of gene therapy vector design intellectual property owned by St Jude Children's Research Hospital and University College London. The authors report no other conflicts of interest in this work.

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