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Review Article

Gene Therapy for Hemophilia: Facts and Quandaries in the 21st Century

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Abstract. Therapy for hemophilia has evolved in the last 40 years from plasma-based concentrates to recombinant proteins and, more recently, to non-factor therapeutics. Along this same timeline, research in adeno-associated viral (AAV) based gene therapy vectors has provided the framework for early phase clinical trials initially for hemophilia B (HB) and now for hemophilia A. Successive lessons learned from early HB trials have paved the way for current advanced phase trials. Nevertheless, questions linger regarding 1) the optimal balance of vector dose to transgene expression, 2) amount and durability of transgene expression required, and 3) long-term safety. Some trials have demonstrated unique findings not seen previously regarding transient elevation of liver enzymes, immunogenicity of the vector capsid, and loss of transgene expression. This review will provide an update on the clinical AAV gene therapy trials in hemophilia and address the questions above. A thoughtful and rationally approached expansion of gene therapy to the clinics would certainly be a welcome addition to the arsenal of options for hemophilia therapy. Further, the global impact of gene therapy could be vastly improved by expanding eligibility to different patient populations and to developing nations. With the advances made to date, it is possible to envision a shift from the early goal of simply increasing life expectancy to a significant improvement in quality of life by reduction in spontaneous bleeding episodes and disease complications.

Keywords: Adeno-associated virus; Gene therapy; Hemophilia.

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Introduction. Hemophilia is a bleeding disorder that results from mutations in the *F8* or *F9* genes encoding coagulation factors VIII (FVIII) or IX (FIX), respectively. Deficiency or dysfunction of these clotting factors disrupts the coagulation system and results in frequent, spontaneous bleeding into the joints leading to chronic arthropathy, the hallmark of severe disease (<1% normal FVIII or FIX activity). In severe disease, people with hemophilia (PwH) are infused intravenously with recombinant or plasma-derived factor concentrates for prophylaxis against joint bleeds.[1] In patients with FVIII deficiency or hemophilia A (HA),

replacement therapy with standard half-life products is required 2-3 times per week, whereas therapy with standard half-life FIX in hemophilia B (HB) is required twice per week. The advent of extended half-life (EHL) products[2,3] has dramatically changed the infusion burden for HB patients to as infrequently as once every 2 weeks, whereas currently licensed EHL FVIII products have not had as dramatic of an increased half-life (up to 1.5-fold) presumably due to the limitations imposed by von Willebrand factor (VWF), the carrier for FVIII in circulation.[4] Recent advances with non-factor therapies (NFTs) that either mimic the cofactor function of activated FVIII (emicizumab)[5,6] or aim to "rebalance" the coagulation system by decreasing natural anticoagulants (antithrombin,[7] tissue factor pathway inhibitor,[8] and protein C[9,10]) are revolutionizing the need for intravenous factor therapy for prophylaxis but at this time cannot be used to treat bleeding episodes (see the chapter by Dr. Makris and Dr. Castaman) and have been associated with thrombotic complications in some patients.[11,12]

Robust preclinical development of a liver-directed gene-based therapeutic approach for hemophilia has culminated in promising clinical trial data by several independent groups (**Figure 1**). These trials support the prospect of a one-time infusion that could modify the hemophilia phenotype, thus offering several potential advantages compared to the current system of therapies. Hemophilia served as a model disease for gene therapy trials due to its monogenic nature, straightforward assessment of the efficacy by measurement of circulating FVIII or FIX levels, and easily quantifiable clinical endpoints such as bleeding rates and consumption of clotting factor concentrates. Further supporting its appeal is the ability to improve outcomes with even the modest efficacy of raising factor levels to >1% (the goal of prophylactic factor replacement). First-in-human gene therapy trials for hemophilia using retroviral or adenoviral vector for liver gene therapy, or non-viral vector-based approaches for skin fibroblast transduction and implantation into the omentum were hampered by limited and transient efficacy and immune responses to some viral vectors.[13] Moreover, the use of integrating murine retroviral vector to genetically modified hematopoietic stem and progenitor cells (HSPC) for some primary immunodeficiencies raised concerns for oncogenicity.[14] The hemophilia clinical studies in progress are based on the use of recombinant adeno-associated viral (rAAV) vectors, which have demonstrated efficacy and safety. In some AAV-based strategies, long-term improvement of the disease phenotype with an excellent safety profile was achieved; these data will be discussed below. Strategies using rAAV vectors targeting skeletal muscle for HB[15,16] or lentiviral vectors for transduction of HSPC for HA[17] are being planned or ongoing, respectively; these will not be discussed here.

AAV-Based Gene Therapy: the Facts. AAV is a non-pathogenic, replication-deficient member of the parvovirus family. Naturally occurring wildtype (WT) AAV consists of a single-stranded DNA genome with two open reading frames flanked by inverted tandem repeats (ITRs).[18] Binding of WT-AAV to heparan sulfate proteoglycans on the host cell allows uptake and,

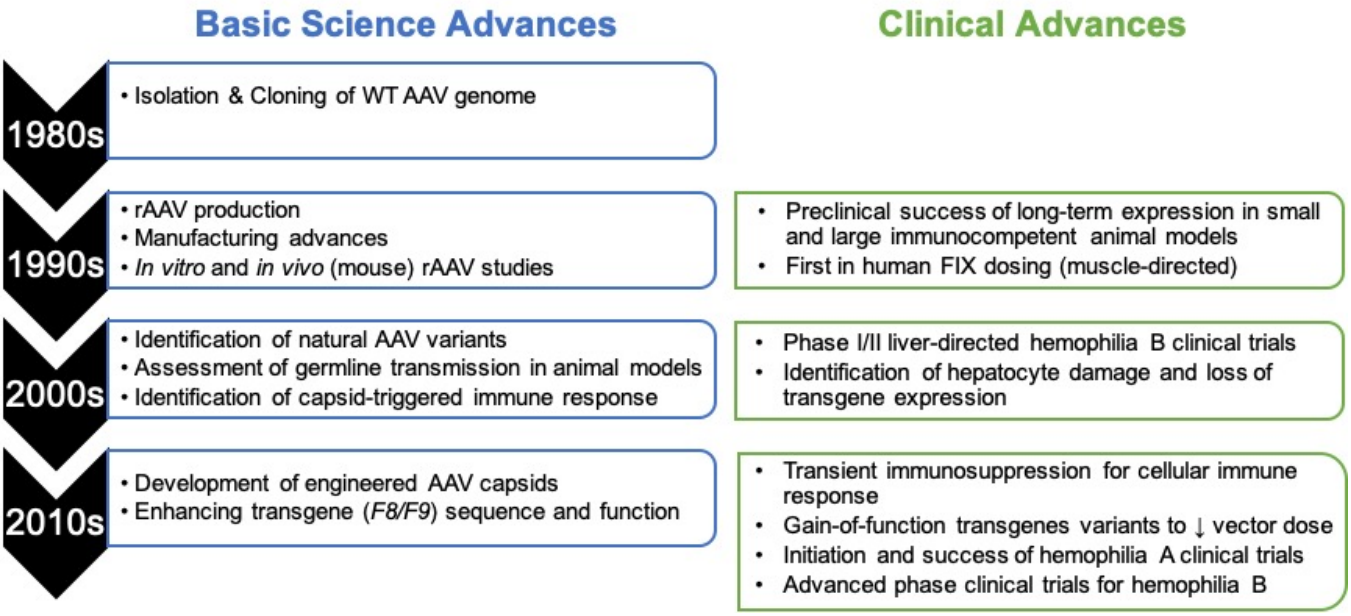

Figure 1. Timeline of evolution of rAAV from basic science advances to clinical gene therapy. A significant body of basic science research allowed for translation from wildtype AAV to the recombinant AAV (rAAV) vectors used today. These included improvements in vector engineering and manufacturing, transgene optimization, and elucidating the immune response to rAAV in humans (as no preclinical model predicted this result). Further, laborious efforts in preclinical toxicity evaluation in the early 2000's allowed clinical trials to advance rapidly in the 2000-2010s without requiring reassessment of these pharmacology/toxicology studies. Clinically, these data have allowed moving from the first-in-human trials in skeletal muscle to phase III trials for both hemophilia A and B with licensure expected in the near future.

replication occurs following the entry into the host nucleus. Integration rates at the AAVS1 site of chromosome 19 (which requires a functional *rep* gene) vary from 45% in HeLa cells (aneuploid cells) to only 2.5% in diploid human fibroblast cells.[19,20] In the rAAV vectors for hemophilia applications, the WT-AAV coding sequences are replaced with an *F8 or F9* transgene under control of a tissue-specific promoter and flanked by ITRs to allow packaging and production of the vector. Although the genomes of the rAAV vectors remain largely episomal[21-24] as they lack the *rep* gene, rAAV vectors may integrate into the host DNA at other sites (reviewed in 24). Thus, the integration pattern and its potential implication for oncogenesis due to rAAV vectors differ from that of WT-AAV. Of the four currently available distinct rAAV production platforms suitable for scaling up vector production,[25] two have been most commonly used for hemophilia gene therapy vectors. Hemophilia rAAV vectors to date have been produced via either transfection of mammalian cells with naked plasmid DNA or introduction of baculovirus expression vectors into *Spodoptera frugiperda* (Sf9) insect cells followed by cell lysis and purification via cesium chloride (CsCl) gradient sedimentation or ion exchange chromatography.[26-30] The capsid-determined tropism of the various AAV serotypes and promoter/enhancer elements used guide transgene production to the target tissue of interest. To date, in hemophilia, this has largely focused on liver-directed transgene expression under the control of a liver-specific promoters/enhancers that restrict expression to hepatocytes.[31,32]

Together, these distinct systems and advances have facilitated the use of rAAV vectors for gene therapy applications in hemophilia. **Tables 1** and **2** summarize the multitude of rAAV-based gene therapy trials for hemophilia A and B, respectively. These contemporary trials are the product of decades of preclinical work and build on the successes and lessons learned from the early gene therapy studies in HB.

AAV gene therapy for HB. Due to the packaging constraints of the rAAV genome, the pioneering rAAV hemophilia gene therapy studies were conducted in HB as the *F9* cDNA is 1.6 kb in size.[33] The evolution of these early trials was guided by both advances in the basic understanding of rAAV and enhancements in vector production (**Figure 1**). The first-in-human rAAV *F9* gene therapy trial utilized a ubiquitous cytomegalovirus (CMV) promoter/enhancer, and the rAAV2 serotype (rAAV2-CMV-*F9*-*WT*) injected into skeletal muscle and demonstrated safe and prolonged local, but not systemic, FIX expression.[34,35] This expression was not hampered by either pre-existing neutralizing antibodies (NAbs) to rAAV2 or a post-infusion immune response to the vector and/or transgene. The excellent short and long-term safety profile of this trial motivated studies targeting the liver (as it is the natural site of FIX production). The trial sponsored by Avigen and Children's Hospital of Philadelphia (CHOP) of rAAV2-*F9* under control of a liver-specific promoter (rAAV2-hAAT-*F9*-*WT*) administered the vector via

Table 1. AAV hemophilia A clinical trials under the control of liver-specific promoter.

Sponsor (ID)	Phase	Serotype	Cell Line	Transgene	Dose (vg/kg)	Enrolled (planned)	FVIII:C$^{\infty}$	# with ↑ ALT	Follow-up (yrs)	NCT Number
Biomarin ("BMN270")	I/II	AAV5	Insect	coBDD-F8	6×10^{12} 2×10^{13} 4×10^{13} 6×10^{13}	1 1 6 7	< 1 < 1 7.9 16.4	1/1 0/1 4/6 6/7	3 3 3 4	02576795
Spark ("SPK-8011")	I/II	AAV-Spark200‡	Mammalian	coBDD-F8	5×10^{11} 1×10^{12} 2×10^{12}	2 3 7	6.9-8.4 5.2-19.8 < 5 – 25	0/2 1/3 5/7	2-3 2-3 2-3	03003533
Pfizer ("SB-525")	I/II	AAV6	Insect	coBDD-F8	9×10^{11} 2×10^{12} 1×10^{13} 3×10^{13}	2 2 2 5	NR 1-2 5-10 64.2	0/2 2/2 0/2 4/5	2 2 2 ~ 1	03061201
UCL/SJCRH ("GO-8")	I	AAV8	Mammalian	coF8-V3	6×10^{11} 2×10^{12} 4×10^{12}	1 3 3	7 8-29 45-74	1/1 1/2 NA	2 1-2 < 1	03001830
Bayer ("BAY2599023")	I/II	AAVhu37	Mammalian	coBDD-F8	5×10^{12} 1×10^{13} 2×10^{13} 4×10^{13}	2 2 2 (2)	~3-10 ~5-15 ~15-70	0/2 NA 2/2	1 < 1 < 1	03588299
Baxalta/Shire ("BAX 888")	I	AAV8	Mammalian	coBDD-F8	2×10^{12} 6×10^{12} 1.8×10^{13}	2 2	NA	NA	NA	03370172

‡Similar to AAV LK03 serotype [71]; $^{\infty}$last available FVIII activity data, chromogenic assay where available listed as range or median (if available).

AAV, adeno-associated virus; ALT, alanine aminotransferase; BDD, B-domain deleted; co, codon-optimized; FVIII, factor VIII; NA: not available, NAb: Neutralizing antibodies; NCT, national clinical trials; SJCRH, St. Jude Children's Research Hospital; UCL, University College of London.

Table 2. AAV hemophilia B clinical trials under the control of a liver-specific promoter.

Sponsor (ID)	Phase	Serotype	Cell Line	Transgene	Dose (vg/kg)	Enrolled n (planned)	FIX:C∞ (median)	# with ↑ ALT	Follow-up (years)	NCT Number
Avigen/CHOP	I/II	AAV2	Mammalian	F9-WT	8×10^{10} 4×10^{11} 2×10^{12}	2 3 2	< 1 < 1 < 1	0/2 1/3 1/2	12-15	00076557
SJCRH/UCL	I	AAV8	Mammalian	co-sc-F9-WT	2×10^{11} 6×10^{11} 2×10^{12}	2 2 6	1.4-2.2 2.1-2.9 2.9-7.2	0/2 0/2 4/6	8	00979238
uniQure (AMT-060)	I/II	AAV5	Insect	coF9-WT	5×10^{12} 2×10^{13}	5 5	1.3-8.2 (5.3) 3.9-11.1 (7.1)	1/5 2/5	4	02396342
Spark ("Spark-9001")	I/II	Spark-100	Mammalian	coF9-Padua	5×10^{11}	10	14-81 (29.5)	2/10	> 3	02484092
Shire ("BAX 335")	I/II	AAV8	Mammalian	co-sc-F9-Padua	2×10^{11} 1×10^{12} 3×10^{12}	2 4 2	3.5* 12.0* 45*	NA NA 2/4	> 2.5	01687608
Uniqure (AMT-061)	IIb	AAV5	Insect	coF9-Padua	2×10^{13}	3	31.3-50.2 (40.8)	0/3	1-2	03489291[Δ]
Freeline ("FLT180a")	I/II	AAVS3	Mammalian	coF9-Padua	4.5×10^{11} 7.5×10^{11} 9.75×10^{11} 1.5×10^{12}	2 2 4 2	37-38 2-60 57-139 90-253	0/2 2/2[ϕ] 1/2[ϕ] 2/2[ϕ]	2 1 < 1 < 1	03369444
Dimension ("DTX 101")	I/II	AAVrh10	Mammalian	coF9-WT	1.6×10^{12} 5×10^{12}	3 3	1.67 22.47	NA 3/3<	< 1	02618915

[∞]last available reported data, aPTT based clot assay values; *median peak FIX values, only two patients with sustained FIX activity beyond 1 year[50]; [ϕ]patients required additional immunosuppression despite prophylactic steroids; [Δ]trial includes patients with neutralizing antibodies to AAV5

AAV, adeno-associated virus; ALT, alanine aminotransferase; co, codon-optimized; FIX, factor IX; NA: not available, NCT, national clinical trials; SJCRH, St. Jude Children's Research Hospital; UCL, University College of London

the hepatic artery in 7 subjects (**Table 2**).[36] The low and mid-dose cohorts were intentionally subtherapeutic. In the high dose cohort ($2x10^{12}$ vg/kg, n=2), pre-existing anti-rAAV2 NAbs did preclude transgene expression in one subject, in contrast to the prior skeletal muscle trial.[35] Another high-dose treated patient initially achieved a FIX level of 11% but lost FIX activity concurrent with a transient rise in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, markers of hepatocyte damage. After several attempts by many groups to develop preclinical models to understand this phenomenon, it became clear that such a complication is observed only in humans.

After consultation with regulatory agencies, another subject (Subject G) was dosed with subtherapeutic vector at 4×10^{11} vg/kg with a planned longitudinal collection of peripheral blood mononuclear cells (PBMCs) to test for potential cellular immune responses to the two neoantigens (rAAV capsid protein and FIX) using a sensitive and specific technique, the interferon-γ (IFNγ) enzyme-linked immunosorbent spot (ELISPOT). These studies suggested that the underlying mechanism of this toxicity is likely a cytotoxic T cell-mediated immune response against the vector capsid sequences displayed on hepatocytes with resultant loss of transduced cells and consequent constraint on transgene expression. There was no evidence of cellular immune responses to FIX. There was, however, a temporal association between the expansion of the rAAV2-capsid cellular response, and a rise in the liver enzymes (ALT/AST).[37]

These data guided the third defining HB trial, led by St. Jude Children's Research Hospital (SJCRH) and University College of London (UCL), which used rAAV8-LP1-*F9-WT* at escalating doses in 10 men with HB.[38,39] This study was the first to 1) infuse an *F9* vector via a peripheral vein (which was made possible by the strong liver tropism of AAV8 compared to AAV2) and 2) demonstrate that initiating prednisone within 48 hours of noting a rise in ALT or drop in FIX could limit the loss of FIX expression in vector-infused patients. Further, this trial supported the dose-dependency of the cellular immune response to the capsid as none of the patients in the low (n=2) or intermediate (n=2) dose cohorts demonstrated an ALT rise (**Table 2**), whereas 4 of 6 subjects (66%) in the high dose cohort showed evidence of a cellular immune response. Over 8 years of follow-up, these high-dose subjects have maintained FIX levels of 2.9-7.2% (**Table 2**).[40]

These studies imparted two critical lessons. First, patients with neutralizing antibodies against the AAV serotype should be excluded to avoid the inhibitory effect on gene expression. Second, as there are no biomarkers that predict the onset of rAAV capsid-mediated cellular response, close monitoring of liver enzymes and factor levels should be used as surrogate markers for the ongoing cellular responses in real-time. Although ELISPOT assays are the most accurate for the diagnosis of T cell responses, the turn-around time of the assay is not ideal due to the need for rapid initiation of

therapeutic intervention to stop or control the loss of transduced cells and transgene expression. Consequently, current clinical trials use the ALT as a biomarker of potential capsid-directed cellular immune response and a hallmark of liver damage; ALT is more sensitive to hepatocyte damage than AST and has a longer half-life.[41]

These studies paved the way for the trial sponsored by Spark Therapeutics, which leveraged the hyperactive *F9* variant, *F9-Padua*, with the goal to decrease the therapeutic vector dose while increasing transgene activity.[42] *F9-Padua* results in an arginine to lysine substitution at position 338 in the FIX protein and has a specific activity (activity to antigen ratio) of > 8.[43] Thus, even small amounts of FIX antigen can provide hemostatically normal FIX activity without increasing the risk of thrombosis[44-46] compared to FIX-WT. A trial of 10 subjects injected with 5 x 10^{11} vg/kg of rAAV-Spark100-*F9-Padua* resulted in sustained FIX activity of ~30% of normal over multiple years of follow-up and only 2/10 (20%) subjects required prednisone therapy for a capsid-mediated immune response, which correlated with a rise in ALT and decline in the FIX activity.[47] Despite decreasing the vector dose 4-fold, a mean FIX activity of 30% was achieved, which is ~15-fold higher than with FIX-WT at a similar dose in the SJCRH trial (**Figure 2**). It should be noted that in the Shire-sponsored rAAV8-F9-Padua trial, loss of transgene expression in the high dose cohort (3 x 10^{12} vg/kg) could not be rescued in all patients,[48-50] which may be due to higher vector doses and/or differences in vector content including CpG islands.[51,52]

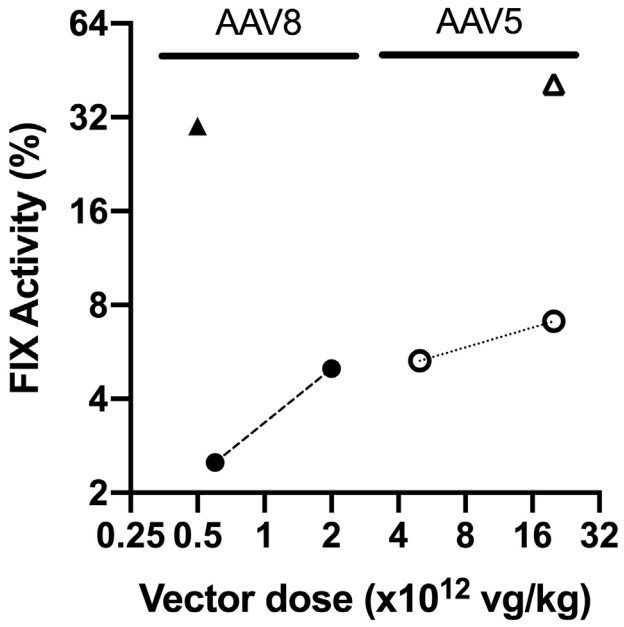

Figure 2. Hemophilia B trial efficacy using *F9* WT versus Padua transgenes. Mean FIX activity is significantly higher using *F9-Padua* (triangles) compared to *F9-WT* (circles) irrespective of AAV8 (closed symbols) or AAV5 (open symbols) vector serotype. Data derived from values listed in **Table 2**.

Data from the Freeline sponsored trial using a novel serotype with liver tropism (AAVS3) encoding *F9-Padua* has recently been presented across four cohorts from 4.5 x 10^{11} to 1.5 x 10^{12} vg/kg. All patients received prophylactic steroids with varying initiation times, depending on the cohort. In the lowest dose group, there was no transaminitis, and FIX levels showed an increase while on steroids with plateau levels at 2 years of 37-38%.[53,54] However, the initial plan to scale up to 1.5 x 10^{12} vg/kg resulted in safety concerns such as transaminitis in both subjects, which required methylprednisolone and tacrolimus to control. One subject expressing supra-therapeutic FIX levels (peak 520%) developed a local thrombotic complication at an arteriovenous fistula when weaned off prophylactic anticoagulation.[55,56] Consequently, the trial suspended this dose, and two additional intermediate-dose cohorts were tested (**Table 2**). All subjects received prophylactic steroid therapy, and tacrolimus was added to control transaminitis either therapeutically or prophylactically. Therapeutic levels of FIX were achieved in both cohorts in a dose-dependent manner (the relatively short follow-up period prevents firm efficacy conclusions at this point).

The uniQure-sponsored trials have utilized an Sf9 insect cell line and baculovirus production system to develop the rAAV5-LP1-*F9* cDNA therapy platform encoding either FIX-WT (AMT-060)[57] or FIX-Padua (AMT-061).[58,59] A potential advantage of rAAV5 is the possibility of a lower prevalence of NAbs in the general population compared to the other AAV serotypes and/or lower avidity of anti-AAV5 NAbs.[60,61] It should be noted that there is a wide range in the reported prevalence of NAbs to AAV, including AAV5, in the general and hemophilia populations due to variability in geographic origin of populations[62-65] but also likely from differences in the assay techniques. Data from uniQure's porphyria gene therapy trial of rAAV5 at 5 x 10^{11} – 1.8 x 10^{13} vg/kg demonstrated a lack of capsid-triggered immune response at those doses; albeit none of the patients had therapeutic transgene levels either.[66] In the AMT-06, 0 trial, 1/5 in the low dose, and 2/5 in the high dose group (2 x 10^{13} vg/kg) had a transient rise in ALT (with onset ranging from 3 to 10 weeks). Still, there was no detectable capsid-mediated cellular immune response as measured by IFN-γ ELISPOT assay (the commonly used surrogate for cellular immunotoxicity) or decrease in FIX levels.[57] The reason for this transient increase in ALT remains unclear, although alcohol consumption and/or antibiotic use were postulated as modulators, in part, of the liver damage. These three patients received steroid therapy, but its therapeutic role is not clear. The median FIX levels in the high dose cohort were 7.1% compared to 5.3% in the low-dose cohort (4-fold lower dose) over 4 years of follow-up, suggesting a lack of clear linear dose-response (**Figure 2**). In the three patients treated with AMT-061 (encoding *F9-Padua*) at

2 x 10^{13} vg/kg, mean FIX activity is similar to the Spark trial with the same transgene at about 40%,[58] but at 40-fold higher dose. As there was seemingly no correlation between the presence of NAbs to rAAV5 and FIX transgene expression in AMT-060,[61] candidates with anti-AAV5 NAbs were not excluded from AMT-061, and the titers of the first three patients are between 1:25 and 1:48.[59] However, this is a preliminary finding, and further assessment of the detection of NAbs and the ability of rAAV5 to overcome the presence of pre-existing NAbs is necessary.

Early phase rAAV-based gene therapy for HB has reached critical mass, providing a firm basis for current phase III clinical trials. Collectively, FIX-Padua across distinct rAAV trials has not shown increased immunogenicity or spontaneous thrombosis, which is consistent with preclinical data in inhibitor-prone HB dogs.[45,46] However, as seen in **Figure 2**, there is a significant variation in the vector dose required to attain similar FIX levels. Of note, the transgenes used in the rAAV5 trials were also used in the rAAV2 and rAAV8 trials. Thus, F9 transgene is unlikely to influence these discrepancies in therapeutic vector doses. The differences in serotype, manufacturing process, post-translational modification of the vector, or combination thereof may be responsible for this discrepancy. A side-by-side comparison of these vector production systems is highly desirable.

AAV Gene Therapy for HA. Although HA is more prevalent than HB, the generation of rAAV vectors that could efficiently accommodate the *F8* cDNA (7 Kb) was challenging due to the limited capacity of the AAV genome at 4.7 kb. Modifications to the *F8* transgene evolved over time. First, the B domain was truncated from > 900 to 14 amino acids as it is not required for full procoagulant activity of FVIII;[67] however, this was not sufficient to allow cloning into rAAV vectors. Subsequently, a series of modifications in the vector design, such as the generation of minimally sized effective liver-specific promoters, enhancers, and other regulatory elements allowed the generation of rAAV vectors that expressed FVIII. Finally, the field developed codon-optimized (co) B-domain deleted (BDD)-*F8* transgene(s) resulting in higher FVIII expression levels without modifications to the amino acid sequence or need for additional space in the transgene.

Several phase I and II HA trials are reporting therapeutic FVIII levels (**Table 1**) in the moderate (1-5%) or mild (> 5%) hemophilia ranges. The vector that is the furthest along the developmental pipeline for HA is Biomarin's BMN-270, which is a rAAV5 vector carrying a co-*BDD-F8* transgene.[68,69] In the high vector dose cohort (6 x 10^{13} vg/kg), median chromogenic FVIII levels were 55% (range 11-95%) in the first year after treatment but, surprisingly, declined over the ensuing four years to 16.4% (**Figure 3A**).[70] Given that there was initially a dramatic increase in FVIII activities, Biomarin also conducted a trial at an intermediate dose cohort (4 x 10^{13} vg/kg). The median chromogenic FVIII activity at year 1 for 5/6 subjects was 24% (one subject had levels < 3%) and over 3 years has declined to 7.9% (**Figure 3A**).[70] The one-stage FVIII activity in these subjects was about 1.6-fold higher than their chromogenic activities (**Figure 3B**). In the high dose cohort, following a rise in ALT in the first patient, all subsequent subjects received prophylactic prednisone. Despite this, all patients still developed a rise in ALT between weeks 3-28, and there was no clear correlation

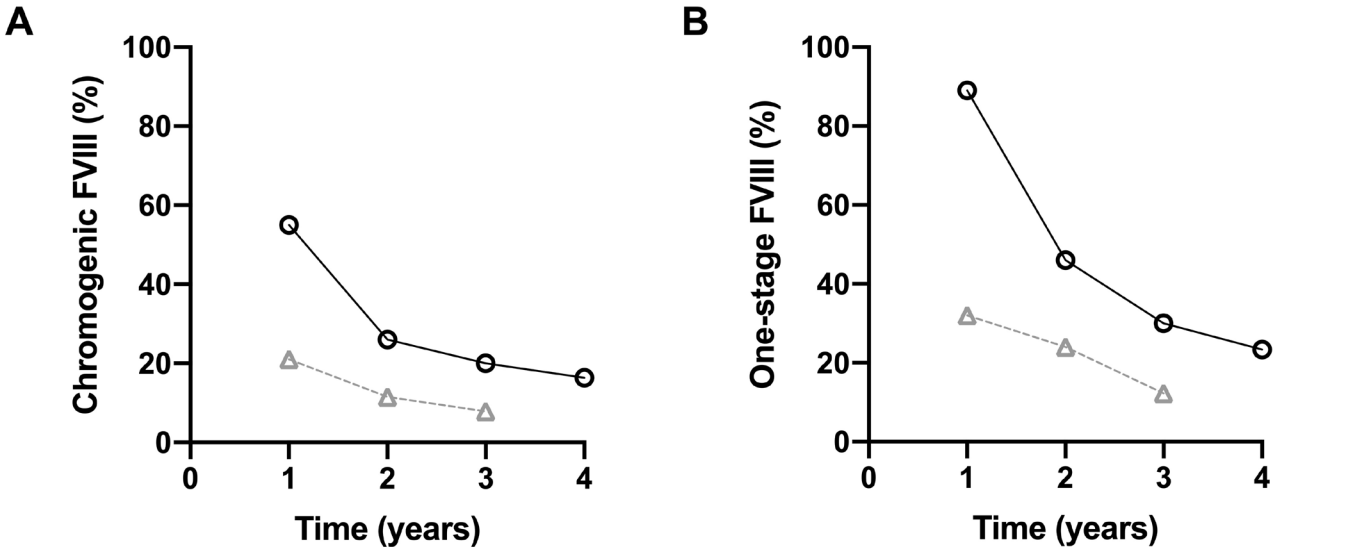

Figure 3. Longitudinal FVIII activity following rAAV5-*F8* infusion in human subjects. A) Median chromogenic FVIII activity over time in the high dose (O, 6 x 10^{13} vg/kg) cohort declined from a median of 55% to 16.4% over four years of follow-up compared to the intermediate dose cohort (Δ, 4 x 10^{13} vg/kg) where levels declined from a median of 21% to 7.9% over three years of follow-up. B) Median one-stage assay based FVIII activity over time in the 6 x 10^{13} (O) and 4 x 10^{13} (Δ) vg/kg rAAV5-*F8* dose cohorts.

between ALT improvement, FVIII activity, and IFNγ ELISPOT results.[69]

In the Spark Therapeutics trial of AAVSpark200-co*BDD-F8* (similar to AAV serotype LK03)[71] within the high dose cohort (2 x 10^{12} vg/kg), five of seven subjects received steroids for either an increase in ALT, declining FVIII levels, or a positive ELISPOT. Of these, two experienced loss of FVIII activity that could not be rescued with prednisone or methylprednisolone with levels falling below 5%.[72,73] In contrast, one subject in the mid-dose cohort (1 x 10^{12} vg/kg) had a rise in ALT, and two of three had a decrease in FVIII (treated with steroids) without an increase in ALT or positive IFNγ ELISPOT. All three have maintained levels of ~5-20%. In the low dose group, neither patient had a rise in ALT or loss of FVIII and have maintained levels of ~7-8%. The UCL-SJCRH sponsored GO-8 trial (rAAV8-*F8-V3*), utilizing a hyperactive FVIII variant (*F8-V3*) with amino acid insertions into the residual B domain sequence,[74] demonstrated elevated ALT in two subjects at the low and mid-dose cohorts (6 x 10^{11} and 2 x 10^{12} vg/kg, respectively) which resolved with prednisone but was not associated with a loss of FVIII activity.[75]

In comparison to these mammalian cell line vectors, the Biomarin trial utilizing an Sf9 insect cell line and baculovirus production system-derived vector required a higher vector dose to achieve therapeutic FVIII levels. For comparison, the low dose cohorts (6 x 10^{12} and 2 x 10^{13} vg/kg) in the Biomarin trial demonstrated < 1-2% FVIII activity (n = 1 per cohort) whereas a log-fold lower dose of 2 x 10^{12} vg/kg in the Spark and UCL/SJCRH trials did result in measurable FVIII activity in the mild hemophilia range for those without an immune response. In a recent publication, the authors argue that the initial delay in achieving plateau FVIII activity levels with rAAV5-*F8* is since the transgene is slightly larger than the vector's packaging capacity and, as the positive and negative DNA strands of the transgene are on separate virions, the full-length functional transcripts take longer to assemble than with other trials,[68] but their findings do not clearly support this. Further, preclinical studies in mouse and canine models with rAAV2[76] or rAAV8[77] carrying the *F8* gene split between two vectors (rAAV vectors carrying the light or heavy chain) delivered simultaneously resulted in similar kinetics of expression to single-chain FVIII. The unexpected loss of transgene activity over time is also unusual in the context of rAAV liver gene therapy. The authors argue that this is due to the turnover of nucleated cells carrying stable full-length episomes, as measured by sequencing analysis of PBMCs; again, these claims are highly speculative at this point.[68]

The decline over time in FVIII levels seen in these subjects in the Biomarin trial has not been observed in the uniQure rAAV5-*F9-WT* trial over 4-years of follow-up despite a 3-fold lower vector dose compared to the Biomarin trial.[78] Further, the FIX expression in ten men with HB injected with rAAV8-*F9-WT* is stable over an extended period of ~ 8 years.[40] Thus, the underlying mechanism of this loss of FVIII transgene expression remains unclear; a combination of vector dose, vector manufacturing, and transgene might impact the stability of the expression levels. At this time, it is too early to identify the best performing rAAV system for HA and long term follow up studies will be required to determine the efficacy of any given strategy.

AAV-Based Gene Therapy: the Quandaries. These significant advances in gene therapy for hemophilia make it likely to enter the clinics in the coming years. There have been no sustained adverse events documented in these trials with follow-up periods ranging from < 3 years to more than 7 years with ongoing observations. However, questions remain about *(1)* target factor level and durability of response, *(2)* long-term follow-up requirements, *(3)* the risk of genotoxicity, (*4)* expanding patient eligibility to inhibitor patients and pediatric population, and *(5)* how to price and pay for gene therapy.

1) Therapeutic transgene expression target and durability of response. Initially, the goal in gene therapy trials was to bring factor levels over the 1% necessary to convert a patient from severe to moderate bleeding phenotype. In some subjects in the SJCRH/UCL and uniQure HB trials, FIX levels of < 3% were not sufficient to prevent joint pain and bleeds, and prophylaxis was necessary.[38,39,57] Further, higher levels are likely required to prevent joint bleeds and stop prophylaxis, as noted by a Dutch pediatric HA study that showed levels > 12% were necessary to prevent joint bleeds.[79] A larger U.S.-based study of adult and pediatric nonsevere HA and HB patients estimated that FVIII or FIX levels > 20% of normal would be needed to prevent hemarthrosis in individuals 25-44 years of age, the typical age of subjects enrolled in early phase gene therapy trials.[80] On the other hand, elevated FVIII (> 150 IU/dL) or FIX (> 129 IU/dL) levels are associated with increased risk for thrombosis compared to the general population.[81-85] Thus, true target FVIII or FIX activity remains debatable.[86]

Understanding the optimal transgene level is essential as the target FVIII or FIX level may affect the choice of vector dose. Recent trials have used fixed doses for all enrolled patients irrespective of underlying joint status or bleeding history. Prior studies have noted variability in bleeding phenotype in patients with severe hemophilia.[87,88] As vectors move from trials to clinical practice, it may be important to consider these modifiers in choosing the appropriate dose for each patient. The dose will need to be carefully balanced against the risk of liver toxicity due to a cellular immune response to the capsid (or unknown mechanisms).[89] At this point, the dose-dependent cellular immune response to the vector

capsid does not correlate with the rise in ALT with insect cell-line derived rAAV5 vectors. Whether this relationship will hold true in the ongoing mammalian vector HA trials is unknown.

2) Long term follow-up requirements. Gene therapy trials to date have typically been very selective in their eligible population. For accurate assessments of both efficacy and safety of a given strategy, long-term follow-up of these subjects is necessary prior to and after approval of a product. Although the vector infusion is given only once, patients will need to be followed for recognition of potential unexpected findings, given the lack of preclinical models that recapitulate the transient liver toxicity due to cellular immune response to vector in humans. In contrast to the loss of transgene activity in the BMN-270 trial, increasing gene expression has been seen in long-term follow-up of a canine HA model,[90] the reasons for these discrepancies continue to be determined. Further, previous retroviral and AAV studies using a CMV promoter in mice and dogs were complicated by gene silencing events of transgene expression;[91,92] this has not been reported with the use of liver-specific promoters.

In addition, adjustments of FVIII or FIX levels may be necessary to accommodate for a subject's physical activity and/or joint status (although vector re-administration at this point is not feasible). Similarly, major trauma or surgery will also require close monitoring and likely transient replacement therapy with factor concentrates. The development of neutralizing alloantibodies ("inhibitors") to FVIII or FIX following gene therapy is an unlikely scenario.[45,46,77,93,94] Still, little is known about subjects with minimal exposure to factor concentrates prior to enrollment in rAAV clinical trials wherein selected persons had > 20 exposure days.

Finally, the risk of germline transmission of viral vectors is a major safety concern. To date, gene therapy has been somatic in nature, and preclinical studies were required by regulatory agencies prior to human trials.[95,96] These did not show AAV in the semen of rabbits or dogs receiving rAAV by intramuscular or portal vein injections, respectively.[97] However, subjects in the rAAV2 trial did have transient detection of vector sequences in the semen.[36,98,99] Subsequent studies in rabbits using intravascular delivery of rAAV vectors were associated with transient detection of a vector in semen in a dose-dependent manner.[100,101] In addition, evidence supported the concept that vector shedding into the semen did not require germ cells, as the semen of vasectomized rabbits (i.e., lacking germ cells) transiently contained vector sequences.[100] Although vector shedding in the majority of the rAAV serotypes tested to date seem consistent with these findings, the risk, if any, of inadvertent dissemination of vector to germ cells needs to be determined after the development of other natural or engineered rAAV vectors as these results may vary due to vector and/or production platforms. The advice of the regulatory agencies is to use barrier contraception while the semen contains rAAV particles.[102]

3) Risk of genotoxicity. In evaluating the safety in terms of potential genotoxicity due to rAAV vectors, it is important to note that the recombinant AAV vector only rarely integrates into the host DNA, whereas the WT-AAV may exhibit latent infection via integration events mediated by the *rep* gene. Thus, the rate and pattern of integration and risk of insertional mutagenesis differ from the WT-AAV. Recombinant AAV vectors are poorly integrating vectors with no preferential specific sites. For rAAV, integration events, if any, occur at diverse locations depending on the experimental model. Thus, findings from WT-AAV studies are not directly relevant to rAAV vectors. Moreover, in some *in vitro* experimental models, WT-AAV2 may, in fact, protect against tumor formation.[103,104] Nevertheless, over the years, sporadic reports on the risk of AAV integration and increased risk of tumor formation raised safety concerns, particularly for genetic diseases with a long life-expectancy such as hemophilia.

In 2001, animal studies using rAAV in neonatal mice with MPS VII, with a high vector dose and a strong enhancer element, demonstrated some integration, which led to hepatocellular carcinoma (HCC).[105,106] Upon discussion with various investigators and regulatory agencies, trials using rAAV vectors for genetic disease were continued but with a commitment to long-term follow up of the subjects who received a direct injection of the vector for ~ 15 years. Some of these studies of rAAV liver gene therapy were presented at scientific meetings, and early evaluation did not show evidence of increased risk of tumor formation.[98] Subsequently, in 2007, a more detailed examination of the molecular evolution of the HCC in neonatal mice showed that integration occurred largely at a miRNA site or the *Rian* locus, which is transcriptionally active in neonatal but not adult mice. This locus is absent in vertebrates except for mice and rats.[104] Additional studies in adult rodents, dogs, and non-human primates could not confirm the increased risk of tumor formation by rAAV vectors.[107-112] These risks can further be mitigated by modulating vector (dose, promoter, enhancer) and subject (age, target tissue) characteristics.[113] Interestingly, the vector constructs adapted for several clinical studies for rAAV liver gene therapy seems to be associated with the least, if any, risk of tumor formation in mouse models.[113]

In 2015, Nault and colleagues showed the integration pattern of WT-AAV in human subjects using a series of tissues from HCC affected and normal areas.[114] In brief, only 11/193 samples showed the integration of AAV in potential genes associated with HCC, but samples were

also positive for viral hepatitis and alcoholic liver disease, and this study lacked data from healthy controls. These findings were again informative of WT-AAV biology but are not necessarily applicable for rAAV used in gene therapy.[103,115,116]

In 2017, Logan and colleagues found that an early rAAV2 vector retained a small WT-AAV2 sequence in the 3' untranslated region (UTR) adjacent to the ITR (derived from AAV2 and used in most rAAV constructs) which contains a binding site of hepatic transcription factors (including HNF1-α).[117] This retained sequence can enhance transcription from the transgene promoter in human hepatocytes and rodent livers. Further, this sequence is captured within the 163-nucleotide frequent insertion region of the WT-AAV2 genome that has been implicated in HCCs. However, there is no definitive proof of insertional gene dysregulation by this sequence. Emerging data presented only in abstract form of HA dogs injected with rAAV carrying canine *F8* showed integration events but without malignant transformation in necropsy samples; the implications of these findings are still unclear.[90] In addition, to date, numerous patients with hemophilia have been treated with rAAV liver-directed gene therapy with no reported significant safety or toxicity concerns;[36,38,39,42,49,57,69,118] long-term data is still being accumulated.

Overall, the likelihood of genotoxicity with rAAV liver-directed gene therapy is likely low. It merits mention that in the > 140 rAAV gene therapy trials targeting a variety of tissues, none have reported oncogenesis. Although WT-AAV infection may be associated with HCC, the risk of rAAV mediated HCC is currently restricted to integration events into a murine-only genetic locus that is active in neonatal mice. However, as shown by recent studies, certain vectors may integrate into the genome indiscriminately. Of note, the long-term follow-up of subjects from the CHOP/Avigen rAAV2-*F9* trial at 12-15 years did not show evidence of tumors via measurement of tumor markers or liver enzymes.[98,119]

Current guidelines from the Food and Drug Administration (FDA) and European Medicines Agency (EMA) recommend a 5-year follow-up period for non-integrating gene therapy vectors,[102,120] such as rAAV, as opposed to prior guidelines which recommended 15 years. The rationale for this shorter term of follow-up may no longer be applicable, especially given the unprecedented fall in FVIII expression from the rAAV5-F8 trial over 4 years of ongoing observation. Further, the coupling in recent trials of gene therapy with immunosuppressive regimens that could modify safety and long-term complications also raises concerns. Consequently, long-term clinical follow-up of patients from these early trials should be undertaken to help inform the safety and efficacy of liver-directed rAAV gene therapy in patients.

4) Expanding patient eligibility. The next step in advancing gene therapy should be to allow for the expansion of the target patient population to those who could benefit from the recent advances in gene therapy. This includes patients historically ineligible for gene therapy trials, including those with inhibitors, patients < 18 years of age or who have fewer factor exposure days, and those with NAbs to AAV serotypes.

4a) Patients with current or prior history of inhibitors to FVIII or FIX. Due to the theoretical concern that gene therapy may increase the risk of inhibitor formation, early phase clinical trials have excluded patients with current inhibitors or a history of inhibitors to FVIII or FIX. To date, data from both HA and HB subjects have not shown any evidence of inhibitor development following gene therapy. AAV liver-directed gene therapy in canine models of HA and HB demonstrate a favorable bias towards inducing immune tolerance to canine FVIII or FIX in inhibitor-prone HA [77] and HB dogs,[110,121] respectively. Our laboratory has shown great promise for gene therapy to provide the dual function of inducing immune tolerance to eradicate inhibitors and provide lifelong endogenous prophylaxis in large animal models of hemophilia A[93] and B.[45] These preclinical studies suggest that the liver-restricted endogenous expression of FVIII or FIX allows for the induction of immune tolerance, at least in part in the HA models, by the upregulation of a regulatory T cell pool.[93] Thus, the possibility of using rAAV liver gene therapy for inhibitor eradication would open a new therapeutic avenue to fulfill an unmet medical need. Inhibitor eradication via immune tolerance induction (ITI) regimens is costly, prone to catheter-related thrombotic and infectious complications in pediatric patients,[122] and is successful in 60-70% of patients with good prognostic risk.[123] In order to maintain tolerance, modern-day practice is to continue factor prophylaxis 2-3 times per week. The use of emicizumab as a prophylactic hemostatic regimen for inhibitor and non-inhibitor HA patients is now largely accepted;[5,6] however, treatment of breakthrough bleeding episodes still requires bypassing agents or FVIII therapy in these patients, respectively. The use of prothrombin complex concentrates in some patients has been associated with thrombosis.[11] However, the desired outcome of standard ITI is the normalization of the hemostatic response to FVIII to avoid bypassing agent therapy and this forms the motivation for definitive inhibitor eradication in inhibitor patients; gene therapy may hold a promising role in this context.[94] To date, one clinical study has been planned to test gene therapy for inhibitor eradication in HA; careful evaluation of preclinical studies in relevant animal models will be critical to support such trials.

4b) Inclusion of pediatric hemophilia patients. The

pediatric patient population could also have tremendous benefit from gene therapy approaches. Routine factor prophylaxis generally requires indwelling central lines in infants and toddlers. The advent of emicizumab and other NFTs, which are being developed to be administered subcutaneously, could thus alleviate the burden in the care of hemophilia. Moreover, EHL FIX products are highly effective in HB patients with reduced frequency of injections,[124] but all of these therapies would still require life-long infusions. Understanding the durability of efficacy and risk of potential adverse events from the different rAAV vector serotypes will allow consideration of the inclusion of young patients in gene therapy trials.

4c) Inclusion of patients with NAbs to AAV capsid proteins. Overall, 20-40% of candidates for intravascular AAV gene therapy are not eligible due to the presence of NAbs to the vector capsid resulting from cross-reactivity after natural exposure to WT-AAV.[125] To date, it has been challenging to compare the efficacy of a given serotype across distinct studies due to a lack of normalization of the assays as most are developed "in house." Potential modifiers of the rates/titers of NAbs could include (1) reporter gene, (2) assay technique whether neutralizing assays, non-neutralizing antibody assays (ELISA) or cellular assays, (3) presence or absence of empty vector capsids, (4) age of the patient [65,126] and longitudinal studies in the same population over time,[63] (5) underlying disease,[62,63,65,126] and (6) vector manufacturing. Some candidates may also test positive for more than one serotype at a time. Further, the amount of antigen (AAV capsid protein) delivered by a gene therapy vector is likely to be orders of magnitude higher than natural infection, and whether these immune responses are similar or not remains to be determined. Finally, although antibodies largely mediate inhibition of vector transduction to the AAV capsid, there is evidence that, despite plasma depletion of IgG, there may still be some inhibitory effect on the vector transduction.

Several attempts aimed at the identification of either naturally occurring or engineered AAV serotypes are in development. However, to date, no candidate serotype allows for vector administration without neutralizing effect or vector re-administration.[127] The latter especially may be needed to rescue transgene expression from either (a) the loss of the transduced cells by liver toxicity or (b) dilution of the transgene expression levels when delivered to young children, as the non-integrating nature of AAV precludes effective transfer of the therapeutic genes to the liver daughter cells upon cell division. To date, there is no clear evidence that such a strategy is highly effective in both preclinical and clinical models. Early efforts, including transient immunosuppression[128] or altering ratio of empty "decoy" capsids,[40,127] have not been successful, but there are some promising strategies. The use of plasmapheresis,[129] catheter-guided perfusion of the portal vein to flush out NAbs,[130] and more recently the use of IgG degrading enzyme of *Streptococcus pyogenes* (IdeS)[131] or *Streptococcus zooepidermicus* (IdeZ)[132] could reduce enough anti-AAV IgG to allow vector efficacy. However, most of these strategies have been tried in the presence of low-titer NAbs (< 1:20); some require invasive procedures, and may have other sequelae such as opportunistic infections.[133] A recent study, employing rapamycin nanoparticles in conjunction with the rAAV vector, allowed for induction of tolerance to that vector serotype in mice and non-human primates, which could permit re-administration.[134] Vector re-administration was also possible in a limited study of IdeS in NHP, but additional safety data regarding anti-IdeS antibody development and the need for readministration is needed. The current lack of sound safety data using any of these strategies in combination with gene therapy precludes firm conclusion on their utility to circumvent anti-AAV NAbs. Although there is some preclinical and clinical evidence that NAbs to AAV5 may not prevent transgene expression,[61] this requires further study as delineated above. **Table 3** outlines a hypothetical strategy for the expansion of hemophilia gene therapy moving forward, and the efficacy and safety outcomes that warrant close monitoring as access is expanded.

5) Price and reimbursement of gene therapy for hemophilia: challenges facing a "one and done" treatment for an orphan disease. Motivated by the emerging success of rAAV liver gene therapy for hemophilia, there is an ongoing debate regarding the cost and payment for clinical gene therapy. A recent study analyzed the cost-effectiveness of a given gene therapy approach compared to standard, uncomplicated prophylaxis in non-inhibitor patients.[135] The Markov Model used by Machin *et al.* assesses disease outcomes (bleeding, surgical intervention, hospitalization) and quality of life against cost in a hypothetical adult HA population on prophylaxis. In this model, the cost of gene therapy was based on the first approved AAV drug in the US, developed for inherited retinal degenerative disease (estimated cost of $850,000). Interestingly, in hemophilia, the gene therapy strategy was considered more cost-effective than protein-based prophylaxis with a superior quality of life performance.

Both payers and the hemophilia community are exploring ways to distribute this cost either on a per annum basis (depending on durability) or with a capped annuity. However, constraints in the existing healthcare systems will need to be overcome to make this a reality. Further, the initial production and development costs are driving current pricing; the hope would be that as gene therapy matures in the clinics, these costs will decline. When this occurs, gene therapy could become

Table 3. Hypothetical strategy general assessment of subjects in early/advanced phase clinical trials for hemophilia.

Proposed staged evaluation of candidates for AAV liver gene therapy in hemophilia				
	Current	**Step 1**	**Step 2**	**Step 3**
Age at enrollment (years)	≥ 18	13-18	13-18	≤ 13
Disease severity	Severe	Severe/Moderate	Severe/Moderate	Severe/Moderate
Inhibitors				
FVIII	No current or prior history of inhibitor	History of transient low titer inhibitor	History of high titer, previous ITI or presence	History of high titer, previous ITI or presence of inhibitor
FIX	No current or prior history of inhibitor	No current or prior history of inhibitor	No current or prior history of inhibitor	No current or prior history of inhibitor
Prior factor exposure	20-50 exposure days	Limited	Limited	Limited
Anti-AAV NAb titer	≤ 1:5	Non-AAV5 ≤ 1:5 AAV5 > 1:5	Non-AAV5 ≤ 1:5 AAV5 > 1:5	Non-AAV5 ≤ 1:5 AAV5 > 1:5
Long term follow-up requirements				
Efficacy Outcomes				
Factor levels	X	X	X	X
ABR and factor consumption	X	X	X	X
Pain/discomfort	X	X	X	X
Mental health and quality of life	X	X	X	X
Safety Outcomes				
Inhibitor	X	X	X	X
Vector shedding (semen)*	X	X	X	N/A
Liver Function tests	X	X	X	X
Screening for cancer**	X	X	X	
Thrombosis		X	X	X

*Safety concern that will establish risk as early patients are eligible.
**Imaging and laboratory (alpha-fetal protein) evaluation. Minimal duration of 5 years post enrollment.

an affordable and reasonable therapy in developing nations as well. Gene therapy affords the enormous potential to alleviate the burden of disease and improve the quality of life for people both in developed and developing countries, but how gene therapy is priced will play a significant role in its global impact.

Conclusions. A copious body of preclinical and clinical work has brought hemophilia gene therapy to the brink of becoming a tangible reality for hemophilia. The phase I/II data show the ability to ameliorate the bleeding phenotype and improve quality of life significantly and have paved the way for phase III trials. Evaluating gene therapy in PwH with pre-existing inhibitors or in pediatric subjects will likely be the next frontier for rAAV. Together with NFTs, gene therapy-based strategies point to a coming transformation in the treatment of hemophilia.

These successes should not minimize the challenges facing the gene therapy research community, including balancing vector dose to limit the cellular immune response while maximizing therapeutic efficacy and understanding the long-term risks from rAAV treatment. Indeed, in most hemophilia trials, the risk of AAV capsid-triggered cellular immune response and/or hepatotoxicity is proportional to the vector dose. This dose-response toxicity should be taken into consideration when assessing the development and choice of a therapeutic approach for genetic diseases with a long life expectancy, such as hemophilia. On the other hand, the dissociation between elevated transaminases and cellular immune response to capsid in recent trials is puzzling and deserves further study. Understanding this finding will require cooperation between industry and academia as differences in vector production, content, serotypes, and/or as yet undisclosed modifications could help explain these discrepancies. Higher vector doses likely carry a higher chance of inadvertent long-term complications. For hemophilia, patients and providers need to consider not only the goal FVIII or FIX expression level but also the amount of vector required to achieve this goal.

In clinical studies in HA, the considerable variability in the FVIII levels following gene therapy and the loss of FVIII levels observed without detection of AAV capsid-triggered immune response is a unique finding with an undetermined mechanism, which might impact long-term efficacy. It appears, at least, that this is not

true for all rAAV vectors as neither uniQure's rAAV5 nor the SJCRH rAAV8 HB trials that have demonstrated a loss of FIX activity over 4 and 8 years of follow-up, respectively.[40,78] Transduction and turnover of non-hepatocytes likely do not contribute to this finding, given the use of a liver-specific promoter. Transient expression outside the liver at very early time points may take place,[136] but this is unlikely to affect long-term persistence. The ability to re-administer vector might become necessary if durable responses are not seen in the current trials. While basic research progress is being made on this front as well, it is not presently a reality in clinical practice.

Further, the risks of integration with or without genotoxicity as well as germline transmission for novel serotypes require further study with long-term follow-up of subjects in the current trials to allow for safe and rational expansion of eligible patient populations, specifically pediatric patients. Finally, development for PwH residing in developing nations should be considered to improve the global burden of hemophilia. Despite several advances in therapeutics for hemophilia, there has not been a significant decrease in the cost of care, which places an undue disease burden on PwH in lower socioeconomic strata. Making hemophilia gene therapy accessible and affordable for all will require advocacy and innovative solutions from the entire community. As the field moves forward and the above questions are answered, gene therapy could undoubtedly be a welcomed therapeutic revolution and provide health equity in hemophilia as proposed by Skinner et al.[137]

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