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INVITED REVIEW ARTICLE

Advances and challenges for hemophilia gene therapy

Paul Batty† and David Lillicrap*

Department of Pathology and Molecular Medicine, Richardson Laboratory, Queen's University, Kingston, Ontario, Canada

*To whom correspondence should be addressed at: Department of Pathology and Molecular Medicine, Richardson Laboratory, Queen's University, 88 Stuart Street, Kingston, Ontario, Canada, K7L 3N6. Tel: +1 6135336342; Fax: +1 6135332907; Email: David.lillicrap@queensu.ca

Abstract

Hemophilia is an X-linked inherited bleeding disorder, resulting from defects in the *F8* (hemophilia A) or *F9* (hemophilia B) genes. Persons with hemophilia have bleeding episodes into the soft tissues and joints, which are treated with self-infusion of factor VIII or IX concentrates. Hemophilia provides an attractive target for gene therapy studies, due to the monogenic nature of these disorders and easily measurable endpoints (factor levels and bleed rates). All successful, pre-clinical and clinical studies to date have utilized recombinant adeno-associated viral (AAV) vectors for factor VIII or IX hepatocyte transduction. Recent clinical data have presented normalization of factor levels in some patients with improvements in bleed rate and quality of life. The main toxicity seen within these studies has been early transient elevation in liver enzymes, with variable effect on transgene expression. Although long-term data are awaited, durable expression has been seen within the hemophilia dog model with no late-toxicity or oncogenesis. There are a number of phase III studies currently recruiting; however, there may be some limitations in translating these data to clinical practice, due to inclusion/exclusion criteria. AAV-based gene therapy is one of a number of novel approaches for treatment of hemophilia with other gene therapy (*in vivo* and *ex vivo*) and non-replacement therapies progressing through clinical trials. Availability of these high-cost novel therapeutics will require evolution of both clinical and financial healthcare services to allow equitable personalization of care for persons with hemophilia.

Introduction

Hemophilia is an X-linked inherited bleeding disorder affecting approximately 196 706 persons globally (1). Hemophilia is caused by mutations in the *F8* (hemophilia A) or *F9* (hemophilia B) genes, resulting in reduced production/function of the factor VIII (FVIII) or factor IX (FIX) proteins. Patients with severe hemophilia have an absence of circulating plasma FVIII or FIX activity (<1%), resulting in spontaneous bleeding affecting the joints and soft tissues. Without treatment, recurrent bleeding results in the development of chronic arthropathy (knee, ankle and elbow) and early mortality. The current 'gold-standard' treatment for persons with hemophilia involves regular self-infusion (prophylaxis) of intravenous concentrates of exogenously derived FVIII or FIX. The aim of prophylaxis is to raise FVIII or FIX activity above a level that is detectable (>1%) to prevent bleeding and reduce or delay the incidence of joint disease (2,3). Due to the short half-lives of these concentrates, this requires patients or their parents to administer infusions, every 2–3 days (hemophilia A) or 3–4 days (hemophilia B). A significant complication of this treatment is the development of anti-drug antibodies (inhibitors) to factor concentrates, resulting in these being ineffective. Due to the significant societal financial costs of factor concentrates (4), there is substantial variation in access to this standard of care globally (1).

†Paul Batty, http://orcid.org/0000-0002-7808-3462
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Novel therapeutics in hemophilia care

Novel therapeutics to improve care for persons with hemophilia are rapidly evolving, including the following: extended half-life FVIII/FIX concentrates, FVIII mimetics (bispecific antibodies), targeting of natural anti-coagulant pathways and gene therapy/editing (5). Half-life extension of FVIII/FIX recombinant concentrates has been obtained by fusion to polyethylene glycol (FVIII & FIX), IgG1-Fc (FVIII & FIX) or albumin (FIX) (6). These modifications have provided significant reduction in dosing for frequency in hemophilia B patients (half-life: 82–102 h), with more modest benefits in hemophilia A (half-life: 14–19 h), due to FVIII's requirement for chaperoning by von Willebrand factor (VWF). Taking this forward, there has been development of recombinant FVIII-Fc bound to the VWF D'D3 binding domain and 2 XTEN linkers (BIVV001). Preliminary data for this agent in the EXTEN-A study (NCT03205163) have described half-life extension to 38 (25 IU/kg) and 44 (65 IU/kg) h, which has potential to significantly change how concentrate prophylaxis is administered in hemophilia A (7). All factor concentrate-based approaches currently require intravenous infusion and will be ineffective in those with FVIII/FIX inhibitors and will result in allo-antibody formation in some patients (~30% in hemophilia A and ~3% in hemophilia B). A recent development, which has caused great excitement within the hemophilia A community, is a subcutaneously administered bispecific monoclonal antibody (Emicizumab) that binds to FIX/IXa and Factor X/Xa and acts as a partial functional mimic to FVIIIa. Phase 3 clinical studies (HAVEN 1–4) of this agent, in adults and children with and without inhibitors, have demonstrated a marked reduction in bleed rates. Further follow-up in post-marketing studies/registries and within 'real-world' settings is, however, required to provide further safety and efficacy data. Novel agents targeting natural anti-coagulants proteins are also in development in clinical (anti-thrombin and tissue factor pathway inhibitor) and preclinical (protein S and activated protein C) studies (5). Despite the number of agents under investigation, all of these approaches will at best result in amelioration of bleeding phenotype, and patients will continue to require conventional factor concentrate in the event of breakthrough bleeds, major trauma or surgery.

Clinical studies utilizing adeno-associated virus-based gene therapy in hemophilia

Cloning of the *F8* (8) and *F9* (9) genes in the early 1980s resulted in rapid translational advances through the development of safe and effective recombinant cell-line derived factor concentrates. Hemophilia subsequently became one of the early targets for gene therapy studies, due to the monogenic nature, with easily measurable laboratory (FVIII/FIX) and clinical (bleed rate) endpoints. Small amounts of transgene expression that might be obtained from gene therapy have the potential to provide substantial clinical benefit, as seen in patients on prophylaxis (>1%) or with non-severe hemophilia (1–40%). Initial clinical gene therapy studies using integrating retroviral, adenoviral and non-viral (*ex vivo*) approaches were associated with transient low-level factor expression (reviewed in 10,11). With concerns, regarding potential immunological complications and oncogenicity of adenoviral/retroviral approaches from other therapy areas, there has been a shift toward usage of 'non-integrating' recombinant adeno-associated viral (AAV) vectors, which form the basis for this short review.

With the limited packaging capacity of AAV vectors (c. 4.7 kb), studies were first conducted in hemophilia B (*F9* cDNA, c. 1.6 kb) (9). The first in-human AAV study utilized an AAV2-FIX vector (2×10^{11} – 1.8×10^{12} vg/kg), delivered by skeletal muscle injection with transient low-level FIX expression (<2%) (12). Despite lack of persistent transgene expression in the plasma, muscle samples from two participants demonstrated FIX-AAV persistence (AAV-FIX DNA or FIX expression) at 3.7 ($n = 1$) and 10 years ($n = 1$) following treatment (13,14). A subsequent study administered this vector by hepatic artery injection (8×10^{10} – 2×10^{12}vg/kg) in seven patients with severe hemophilia B, demonstrating transient FIX expression (peak FIX:C 11%) in one patient treated in the high-dose (2×10^{12} vg/kg) cohort (15). Loss of FIX expression occurred in this patient, coinciding with a transient asymptomatic elevation in liver transaminases, 4 weeks following vector infusion. Intermediate dose treatment (4×10^{11} vg/kg) in a final study participant demonstrated similar milder transaminase elevation, associated with a capsid mediated T-cell response. The first successful AAV-FIX-based gene therapy study (NCT00979238) was conducted by the St Jude Children's Research Hospital and University College London research groups. Recombinant AAV8 containing codon-optimized (co) *F9* (scAAV2/8-LP1-hFIXco) (16,17) was administered to 10 severe hemophilia B patients (2×10^{11}, 6×10^{11} & 2×10^{12} vg/kg). Persistent FIX levels were seen in all individuals (1–6%) after 3.2 years, with 90% reduction of bleeding episodes in the year before (15.5, interquartile range [IQR] 10.3–19.3) and after (1.5, IQR 1.0–4.0) treatment. Treatment responses were dose dependent, with the highest FIX expression in the high-dose cohort (FIX 5.1 ± 1.7%). Elevation in alanine aminotransferase (ALT) occurred in 4/6 patients (weeks 7–10) in the high-dose cohort, with an associated capsid mediated cellular immune response. This was managed with early intervention with corticosteroids, albeit with reduction (50–70%) in FIX expression (17). Long-term follow-up of this cohort (6.7 ± 1.0 years) has described stable FIX expression in all three dosing cohorts (1.9 ± 0.6, 2.3 ± 0.3, 5.1 ± 1.4 IU/dL) and no late toxicity (18). Similar results have been reported using a baculovirus derived AAV5-co-FIX (AMT-060, NCT02396342) in 10 adults with moderate/severe hemophilia B (FIX≤2%) (19). FIX levels of 4.4 IU/dL and 6.9 IU/dL were seen in those treated with either 5×10^{12} (low dose, $n = 5$) or 2×10^{13} vg/kg (high dose, $n = 5$), respectively, with reduction in spontaneous bleed rates. Mild elevations in ALT occurred in three individuals without a capsid specific T-cell response or affecting FIX expression. Stable FIX expression has been reported at 2.5 years, 4.9% (low dose) and 7.4% (high dose), with ongoing bleed reduction and no further adverse events (20). To increase FIX expression, recent trials have incorporated a naturally occurring gain-of-function single nucleotide variation (R338L, FIX-Padua) (21), following on from studies in severe hemophilia B murine (22) and dog (23) models. Ten men with moderate/severe hemophilia B (FIX≤2%) were treated using a bioengineered AAV capsid (AAV-Spark100) containing the FIX-Padua mutation within the expression cassette (NCT02484092) (24). Elevation to a steady-state FIX activity of 33.7 ± 18.5% (normal range 50–150%) was seen at 14 weeks, with significant reduction in annualized bleed rate (pre: 11.1 versus post: 0.4). Asymptomatic elevation in ALT was seen in two patients with detectable cellular immune response to capsid peptides, requiring initiation of prednisolone. Reduction in FIX expression occurred in one of these patients (48% to 18%). Longer-term follow-up (≥5 years) for safety and efficacy in this cohort is ongoing (NCT03307980). Early data have recently been presented for FLT-180a (NCT03369444), a synthetic AAV capsid containing AAV-co-FIX-Padua for two participants treated with 4.5×10^{11}vg/kg, with FIX > 40% at 6–9 months and

no elevation in liver enzymes (25). A number of phases 2 and 3 FIX studies are ongoing (Table 1).

Clinical studies in hemophilia A have been slower despite a higher global prevalence, due in part to the size of the *F8* gene (c. 7 kb). Truncation of the F8 cDNA, removing sequence encoding a haemostatically non-functional domain (B-domain deletion, BDD), has allowed incorporation into AAV vectors. The first successful application of this approach was reported in 2017, using an AAV5-co-BDD-FVIII vector (BMN270, NCT02576795) in a dose-escalation study in nine patients (26). Within the high-dose cohort ($n=7$, 6×10^{13}vg/kg), elevation of FVIII to >5 IU/dL was seen by weeks 2–9, rising into the normal range (FVIII > 50 IU/dL) in 6/7 patients after week 20. FVIII expression was stable at 1 year (median FVIII 77 IU/dL, range 19–164 IU/dL), with reduction in annualized bleed rate (16 to 1 event/year). Elevation in ALT levels occurred in 8/9 participants, without an associated cellular immune response, managed with corticosteroids without change in FVIII transgene expression. An update at 2 years described ongoing bleed reduction (median: 0 events), improvement in quality of life, albeit with lower FVIII levels (mean: 59 IU/dL) (27). Further data are awaited to see if there is ongoing reduction in FVIII activity with time. Preliminary data were recently presented for the SPK-8011 (NCT03003533) and GO-8 (NCT03001830, $n=4$) studies, both using a BDD-FVIII expression cassette from a HEK293T system (28,29). The SPK-8011 study reported mean FVIII levels at 12 weeks of 13% (5×10^{11}, $n=2$), 15% (1×10^{12}, $n=3$) and 30% (2×10^{12}, $n=7$) (28). Elevation in ALT/AST occurred in 4/12 patients, with one requiring in-patient treatment with high-dose intravenous corticosteroids. Two patients in the high-dose cohort lost the majority of FVIII expression in association with a capsid cellular immune response. Further data are awaited, with patients being monitored within an extension study (NCT03432520). Early data from the GO-8 study presented FVIII levels of 7% in a low-dose cohort (6×10^{11} vg/kg, $n=1$), with variable levels in the intermediate dose cohort of 7–63% (2×10^{12} vg/kg, $n=3$) (29). Transient transaminitis occurred in three patients responsive to corticosteroids. In April 2019, Sangamo therapeutics released data from the first eight patients, in the SB-525 study (NCT03061201) using an AAV6-BDD-FVIII vector (30). Dose-dependent FVIII elevation was seen, with 'clinically relevant' increases in the 1×10^{13} and 3×10^{13} dose cohorts. Normal FVIII levels (140 and 94%) were seen at week 6 in two patients treated using 3×10^{13} vg/kg. A number of phase 1–3 studies are currently ongoing (Table 1).

Humoral immunity to AAV and FVIII and FIX

Pre-existing humoral capsid immunity is considered a limitation to successful transduction from systemically administered AAV vectors. As wild-type AAV infection occurs during childhood, this has resulted in exclusion of many patients from recent studies. Geographically variable seroprevalence (and cross-reactivity) to the different AAV serotypes has been reported (31,32). Neutralizing antibodies have been reported in 25% (AAV5) and 28% (AAV8) United Kingdom hemophilia patients ($n=100$), using a luciferase-based *in-vitro* transduction assay, with increased prevalence in those who have received plasma-based therapies (33). Challenging the dogma of lack of successful transduction in the presence of neutralizing antibody (nAb), baseline samples were reanalyzed for anti-AAV5 antibodies, in clinical/preclinical studies using AMT-060 (AAV-FIX) with a luciferase assay. Antibodies not detected using a green fluorescent protein-based assay were detected in 3/10 patients and all non-human primates, which did not impair successful vector transduction (34). These findings are being followed up in a study (AMT-061, NCT03489291), using this AAV5 vector (2×10^{13} vg/kg) incorporating the FIX-Padua variant, with FIX levels of 25–51% described at early follow-up in the first three patients, not limited by pre-existing nAb (35). Further studies in patients with nAb for FVIII (BMN-270-203, NCT03520712) and FIX (HOPE-B: AMT-061-02, NCT03569891) are ongoing. All studies have reported development of capsid antibodies following treatment with AAV, which are present for many years following treatment (18). With the potential for loss of transgene expression with time, there is a need to investigate strategies to modulate the humoral immune response at time of initial treatment or have alternative, non-cross reacting vectors for repeat treatments. Although repeat dosing has successfully been utilized in the canine hemophilia A model (36), whether additional toxicity will be observed following repeated hepatocyte targeting using viral vectors is not known.

There have been no reported inhibitors to FVIII or FIX in clinical AAV gene therapy studies. These studies, however, have only included patients at low risk of antibody formation. Whether inhibitor formation may occur following gene therapy in treatment naïve patients is not known. In contrast to these clinical data, inhibitor formation has been reported in hemophilia A and B dogs, treated with AAV delivered canine FVIII/FIX (37). In the hemophilia A model, one dog from the University of North Carolina colony treated with peripherally administered AAV8-cFVIII developed a transient (<2 months) inhibitor (38), and two dogs from the Queen's University colony treated with portal vein administered AAV2 & 8 developed transient (<5 weeks) inhibitors (Batty, P., unpublished data). In the hemophilia B dog model, a single high-titer FIX inhibitor occurred 5 weeks after following portal vein infusion, associated with fatal intra-cranial hemorrhage. Concerning the FVIII immune response occurring following portal vein infusion, whether this was a result of the AAV-FVIII transgene or from plasma infusion used at the time of surgery is not known. Interestingly, data from canine and murine hemophilia models have suggested that AAV-derived gene therapy may provide immunological tolerance to FVIII/FIX. There is a plan to study (SPK-8016, NCT03734588) whether this may provide a therapeutic method to inducing tolerance to FVIII in patients with FVIII inhibitors.

Safety of AAV-based gene therapy

The main toxicity seen in clinical studies has been dose-related elevation in liver transaminase following vector infusion. Within some, although not all studies, this coincides with demonstration of cell-mediated AAV capsid immunity. Most studies have used either early intervention or prophylaxis with corticosteroids with the aim of protecting transduced hepatocytes. Although the majority of episodes have been managed effectively with this approach, some episodes have been associated with partial (17,24,28) or complete (39) loss of transgene expression, despite intervention. Further published details of studies presented in abstract format with loss of vector transduction are awaited (39,40). Although this toxicity coincides with a detectable anti-AAV capsid T-cell response in some studies, these findings are not consistent. Some patients develop elevation in transaminases without a capsid response (19,27) or a capsid response without elevation in transaminase levels (16,17). The mechanisms underlying this response are likely complex and may involve contributions from factors such as the unfolded protein response.

Table 1. Active hemophilia A & B AAV gene therapy clinical studies (43)

Sponsor Factor IX	NCT	Study	AAV	Vector Dose (vg/kg)	Phase	Patients (target)	ED	AAV Ab	Status
SJCRH/UCL	NCT00979238	n/a	scAAV2/8-LP1-FIXco-wt	2e11, 6e11, 2e12	1	14	≥50	Negative	Not recruiting
Shire (Baxalta)	NCT01687608	AskBio009	AAV8.sc-TTR-FIXco-Padua	2e11, 1e12, 3e12	1, 2	30	n/a	Negative	Not recruiting
Uniqure	NCT02396342	AMT-060-01	AAV5-FIXco-wt	5e12, 2e13	1, 2	10	>150	Negative	Not recruiting
	NCT03489291	AMT-061-01	AAV5-FIXco-Padua	2e13	2	3	>20	Included	Not recruiting
	NCT03569891	AMT-061-02 (HOPE-B)	AAV5-FIXco-Padua	2e13	3	56	>150	Included	Recruiting
Pfizer (Spark)	NCT02484092	SPK-9001-101	AAV-SPARK100-FIXco-Padua	5e11	2	15	≥50	Negative	Not recruiting
	NCT03307980	SPK-9001-LTFU-101		SPK-9001 extension study	2	20	n/a	n/a	Recruiting
	NCT03861273	BENEGENE-2		n/a	3	55	≥50	< 1:1	Not yet recruiting
UCL Freeline	NCT03369444	FLT-180a	AAVS3-FIXco-Padua	6e11, 2e12	1	18	>150	Negative	Recruiting
	NCT03641703			FLT180a extension study	2, 3	50	n/a	n/a	Recruiting
Factor VIII									
Biomarin	NCT02576795	BMN-270-201	AAV5-FVIII-BDD	6e12, 2e13, 6e13	1, 2	15	>150	Negative	Not recruiting
	NCT03392974	BMN-270-302		4e13	3	40	>150	Negative	Recruiting
	NCT03370913	BMN-270-301		6e13	3	130	>150	Negative	Recruiting
	NCT03520712	BMN-270-203		6e13	1, 2	10	>150	Included	By invitation
Spark	NCT03003533	SPK-8011-101	AAV-SPARK200-FVIII-BDD	5e11, 1e12, 2e12	1, 2	30	>150	Negative	Recruiting
	NCT03432520	SPK-8011-LTFU	n/a	SPK-8011 extension study	1,2	100	n/a	n/a	By invitation
	NCT03734588	SPK-8016-101		Dose finding pre FVIII inhibitor study	1, 2	30	> 150	Negative	Recruiting
UCL	NCT03001830	GO-8	AAV2/8-HLP-FVIII-V3	6e11, 2e12, 6e12	1	18	>50	Negative	Recruiting
Sangamo	NCT03061201	SB-525-1603	AAV2/6-FVIII-BDD	9e11, 2e12, 1e13, 3e13	1, 2	20	>150	Negative	Recruiting
Shire (Baxalta)	NCT03370172	BAX-888	AAV8-FVIII-BDD	n/a	1, 2	10	>150	<1:5	Recruiting
Bayer	NCT03588299	BAY2599023 (DTX201)	n/a	n/a	1, 2	18	>150	Negative	Recruiting

Ab, antibody; co, codon optimized; ED, exposure days to factor concentrate; sc, self-complementary; SJCRH, St. Jude Children's Research Hospital; UCL, University College London; vg, vector genomes; wt, wild-type; FVIII-BDD, B-domain deleted FVIII.

Although current understanding is that AAV forms a largely non-integrating vector, whether there is potential for late toxicity is uncertain. To date, the longest follow-up in human studies is 6.7 ± 1.0 years; with durable FIX expression and no liver injury on laboratory testing or ultrasound (18). Long-term data within the hemophilia B dog model reported normal liver biochemistry (5.5–6 years), and radiological appearances (computerized tomography & magnetic resonance imaging) appearances. Liver biopsies demonstrated normal architecture with centrilobular/midzonal hepatocellular hypertrophy, no evidence of malignancy and no detectable integration by linear amplification-mediated polymerase chain reaction (41). Similar findings have been reported after 10 years, from AAV-treated hemophilia A dogs from the University of North Carolina colony with normal liver function and no evidence of malignancy at necropsy (38,42). With limited long-term human data, there is a need for long-term safety monitoring within extension studies and international registries.

Non-AAV-based gene therapy and gene editing. With pre-existing AAV humoral immunity and the potential loss of vector transduction with time, there is a requirement for other viral and non-viral-based vectors for transgene delivery. The predominant methods studied in hemophilia have used lentiviral vectors, which have lower incidence of pre-existing humoral immunity and greater packaging capacity than AAV vectors. Pre-clinical studies using *in-vivo* or *ex-vivo* stem cell (haematopoietic or induced pluripotent) transduction or blood outgrowth endothelial cells have been recently reviewed (43). Two phase 1 studies are registered with plans to evaluate *ex-vivo* lentiviral stem cell transduction for FVIII (NCT03818763) or FVIII/FIX (NCT03217032, YUVA-GT-F801 or YUVA-GT-F901) (44). Data from studies using gene editing to provide more targeted correction are ongoing. Studies have successfully utilized CRISPR/Cas9 using adenoviral (45), AAV (46–48) and non-viral (49) delivery, for correction/amelioration of phenotype in murine hemophilia B models. *In-vitro* data have also been presented using a transcription activator-like effector nuclease strategy, demonstrating correction of the intron-22 inversion in hemophilia A induced pluripotent stem cells (50). The most advanced approach, however, to genome editing in hemophilia is usage of zinc finger nuclease (ZFN), with data presented in the hemophilia B mouse model (51–53). A phase 1 study (SB-FIX, NCT02695160) is currently recruiting using AAV-directed correction using ZFN targeted insertion of the *F9* gene into the first intron of the albumin locus. The aim of this study is to recruit 12 patients, with severe hemophilia B (FIX < 1%), to three dosing cohorts, with the first patient being treated in December 2018 (54).

Clinical challenges to delivery of gene therapy. There is equipoise within the hemophilia community, as to how outcomes should be measured within gene therapy studies, whether using clinical, patient reported or laboratory outcomes. Within this, if factor levels are used as this measure, it is also unclear what level should be defined as success, be this normal (>50%) or within the mild hemophilia (5–40%) range. Given the variability seen in factor level obtained between individuals and studies, if normalization is used as the criterion for success, this may affect licensing decisions around these products. The complexity and number of novel therapeutics, which will become available for treatment of hemophilia, will require alterations in current pathways for the delivery of patient care. Clinicians may require additional training to allow patients to make informed decisions, regarding these novel treatments and balance risk with current uncertainties. A survey conducted in 2018 (1129 physicians) demonstrated significant deficits in understanding of clinical hemophilia gene therapy studies and vector biology (55), which could be improved using an online continuing medical education program (56). Additional support for patients from hemophilia advocacy groups and the pharmaceutical industry will be required to provide accurate information on treatment choices.

Conclusions

The landscape of available treatments for persons with hemophilia is rapidly evolving. Novel replacement therapies are undergoing clinical trials in parallel with gene replacement/modification strategies. Although, the choice of therapy available in the future may in part be directed by financial implications, this may, with time, allow for more personalized care with improvement in quality of life for the next generation of patients with hemophilia.

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