Molecular Therapy
Methods & Clinical Development
Original Article

AAV gene therapy in companion dogs with severe hemophilia: Real-world long-term data on immunogenicity, efficacy, and quality of life

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The hemophilias are the most common severe inherited bleeding disorders and are caused by deficiency of clotting factor (F) VIII (hemophilia A) or FIX (hemophilia B). The resultant bleeding predisposition significantly increases morbidity and mortality. The ability to improve the bleeding phenotype with modest increases in clotting factor levels has enabled the development and regulatory approval of adeno-associated viral (AAV) vector gene therapies for people with hemophilia A and B. The canine hemophilia model has proven to be one of the best predictors of therapeutic response in humans. Here, we report long-term follow-up of 12 companion dogs with severe hemophilia that were treated in a real-world setting with AAV gene therapy. Despite more baseline bleeding than in research dogs, companion dogs demonstrated a 94% decrease in bleeding rates and 61% improvement in quality of life over a median of 4.1 years (range 2.6–8.9). No new anti-transgene immune responses were detected; one dog with a pre-existing anti-FVIII inhibitor achieved immune tolerance with gene therapy. Two dogs expressing 1%–5% FVIII post gene therapy experienced fatal bleeding events. These data suggest AAV liver-directed gene therapy is efficacious in a real-world setting but should target expression >5% and closely monitor those with levels in the 1%–5% range.

INTRODUCTION

Hemophilia is the most common severe inherited bleeding disorder in humans.[1] Hemophilia A (HA), caused by factor VIII (FVIII) deficiency, is more common than hemophilia B (HB), which results from factor IX (FIX) deficiency. Although hemophilia is an X-linked disorder that primarily affects males, females can experience bleeding with low coagulation factor levels resulting from skewed lyonization, hemizygous state due to lack of a second X chromosome, or homozygous inheritance. Bleeding phenotypes correlate with the degree of coagulation factor deficiency with levels <1% of normal (severe hemophilia) resulting in repetitive bleeding episodes into the joints, termed hemarthrosis, or intramuscular, retroperitoneal, or intracranial hemorrhages that can either be spontaneous or trauma induced. Patients with moderate disease (1%–5% normal factor activity) have some spontaneous bleeds but more trauma-induced bleeding and those with mild disease (6%–40%) generally only experience bleeding with trauma. This wide therapeutic window makes hemophilia an optimal gene therapy target as raising the factor activity even to 1%–5% significantly ameliorates the bleeding phenotype[2] with higher levels in the mild or normal (40%–150%) range, further improving disease-related morbidity and mortality.

Several research animal models of hemophilia have been used in preclinical gene therapy studies, including mice, rats, dogs, and nonhuman primates.[3] One advantage of the canine model is the ability to deliver species-specific *F8* and *F9* transgenes, which limits the xenoprotein immune response seen in other model systems. Hemophilia naturally occurs in dogs with genotypic, phenotypic, and immunologic characteristics that mimic humans.[4,5] Prior studies have evaluated the safety and efficacy of liver-directed adeno-associated virus (AAV) vector-mediated gene therapy for HA and HB in dogs housed in research colonies.[6–10] Two recent canine studies demonstrated long-term efficacy of B-domain deleted canine FVIII (BDD-cFVIII) gene therapy in HA colonies housed at Queens University[6] and the University of North Carolina at Chapel Hill (UNC).[7] Both studies used multiple AAV serotypes with varying vector doses and demonstrated durable cFVIII expression with a significant reduction in annualized bleeding rates (ABR). Canine hemophilia studies laid the groundwork for initiating AAV gene therapy clinical trials for HA and HB. AAV gene therapy products for FIX[11,12] and FVIII[13,14] have recently received regulatory approval based on short-term safety and efficacy data demonstrating significant improvement in ABR and quality of life (QOL).

Received 18 October 2023; accepted 30 January 2024;
https://doi.org/10.1016/j.omtm.2024.101205.

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Table 1. Baseline characteristics of companion dogs with severe hemophilia

Dog	Breed	HA/HB	Mutation	Weight (kg)	AAV8 NAb titer	Age (y)	ABR	Vector	Antigen (ng/mL)	Vector dose (vg/kg)	Follow-up (y)
PC1	Shepherd mix	HA	Thr62Met	20.5	<1:3	1.1	11.1	AAV8-BDD-cFVIII-LC + AAV8-BDD-cFVIII-HC	3.2	5×10^{13}	8.88
PC2	Staffordshire terrier	HA	Intron 22 inversion	31	<1:3	2.3	5.6	AAV8-BDD-cFVIII-LC + AAV8-BDD-cFVIII-HC	0	5×10^{13}	8.49[a]
PC3	French bull terrier	HA	Intron 22 inversion	9.5	1:1	0.9	17.3	AAV8-BDD-cFVIII-ΔF	0	6×10^{12}	2.56
PC5	Mixed	HA	Intron 22 inversion	13	<1:5	0.7	18.9	AAV8-BDD-cFVIII-ΔF	1.7	6×10^{12}	5.55[a]
PC6	Mixed	HA	Intron 22 inversion	23	<1:5	1.2	12.8	AAV8-BDD-cFVIII-ΔF + V3	9.6	5.4×10^{12}	5.03[a]
PC7	Labradoodle	HA	Intron 22 inversion	5.8	<1:5	1.3	4.5	AAV8-BDD-cFVIII-ΔF + V3	0	6×10^{12}	4.34[a]
PC8	Golden retriever	HA	Complex[b]	35.2	<1:1	3.8	16.0	AAV8-BDD-cFVIII-ΔF + V3	1.1	6×10^{12}	3.41[c]
PC9	Mixed	HA	Exon 14 10-bp deletion	24	<1:3	0.8	7.8	AAV8-BDD-cFVIII-ΔF + V3	0	6×10^{12}	3.29
PC10[d]	Mixed	HA	Exon 14 10bp deletion	16	<1:3	0.8	2.6	AAV8-BDD-cFVIII-ΔF + V3	0.6	9×10^{12}	3.86[a]
PC11	Cocker spaniel	HA	Intron 22 inversion	18.4	<1:1	5.2	4.5	AAV8-BDD-cFVIII-ΔF + V3	0	6×10^{12}	3.77[a]
PC12	Shih tzu	HA	Intron 22 inversion	8.2	<1:3	1.0	4.0	AAV8-BDD-cFVIII-ΔF + V3	47.8	6×10^{12}	3.84[a]
PC4	Boston terrier	HB	Gly12Arg; Ala28Thr	6.2	1:1	4.7	2.6	AAV8-cFIX-WT	7.7	3×10^{12}	6.04[a]

ABR: annualized bleeding rate.
[a]Follow-up ongoing.
[b]ex18 insertion poly (A) tract, 14nt duplication, p.Y1987Stop.
[c]Lost to follow-up for specimens, follow-up is based upon clinical information provided by owner.
[d]Homozygous female.

As these approved therapies move into the clinical realm, it remains to be seen if these safety and efficacy metrics will be sustained.[15] Indeed, the long-term durability of transgene FVIII remains a central question of this approach. We previously reported 2 years of follow-up on two companion dogs treated with dual AAV8 vectors carrying BDD-cFVIII heavy (HC) or light chains (LC)[16] at a dose of 2.5×10^{13} vg/kg for each vector. Here, we report long-term follow-up data from these dogs and an additional nine severe HA companion dogs and one severe HB companion dog. As these companion animals were not housed within a colony and experienced real-world challenges with regard to exposures and trauma, their responses to AAV gene therapy may better recapitulate non-trial experiences for patients and inform on expected safety and efficacy metrics as well as optimal factor expression levels.

RESULTS

Baseline characteristics

Twelve hemophilic dogs were treated with AAV8 serotype liver-directed gene therapy vectors; 11 of 12 had severe hemophilia A and one of 12 had severe hemophilia B (Table 1). The dogs were of various breeds and weighed between 5.8 and 35 kg at time of vector administration. All dogs had cFVIII or cFIX activity <1% of normal prior to gene therapy. One severe HA dog was female (PC10), all other dogs were male. Intron 22 inversions of the *F8* gene, which is the most common *F8* mutation in humans with severe HA,[17] were found in 7 of 11 (63.6%) severe HA dogs, including PC2 in whom this inversion was detected with updated methodology. Two dogs (PC9 and PC10, littermates) had a 10-base pair (bp) deletion in exon 14 of *F8*. PC8 had multiple genetic changes including a 14-nucleotide duplication and insertion of poly(A) in exon 18. PC1 had a Thr62Met mutation resulting in loss of cFVIII expression. These other *F8* mutations have not previously been described in dogs with hemophilia but resemble the genetic heterogeneity of humans with severe HA. Of the HA dogs, cFVIII antigen was detectable at baseline in PC8 and PC1 (1–3 ng/mL) and 3/7 of the intron 22 inversion dogs but not the 10-bp deletion (n = 2) dogs. The HB dog PC4 had two missense mutations resulting in amino acid changes of Gly12Arg and Ala28Thr and loss of FIX activity but did have detectable FIX antigen.

The median age at time of vector administration was 1.1 years (range 0.7–5.2 years). The median ABR, derived by dividing the number of bleeding episodes prior to gene therapy by the age at gene therapy, was 6.7 bleeds per year with a range of 2.6–18.9. The bleeding episodes prior to gene therapy were treated with plasma, cryoprecipitate, antifibrinolytics, or supportive care measures at the discretion of the treating veterinarian and are summarized in Tables S1 and S2. PC3 received six transfusions of cryoprecipitate for bleeding events prior to vector administration, which resulted in the development of an anti-cFVIII inhibitory antibody with a baseline titer of 21 BU/mL. PC3 was the only dog with a pre-existing anti-cFVIII or cFIX immune response. All dogs had anti-AAV8 neutralizing antibody titers <1:5 prior to gene therapy (Table 1).

Efficacy of AAV gene therapy for hemophilia in companion dogs

Factor activity and antigen expression

Three B-domain deleted (BDD) codon optimized cFVIII constructs were used in this study. An early approach was administration of two distinct vectors for expression of the cFVIII light chain (LC) and heavy chain (HC) each under the control of a hepatocyte-specific

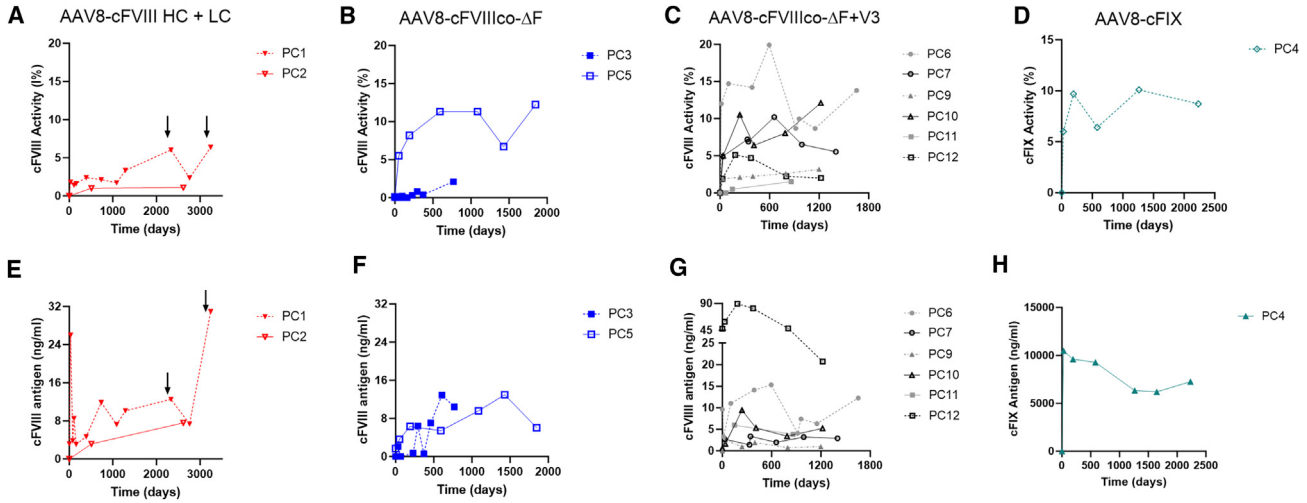

Figure 1. Canine FVIII or FIX activity and antigen following gene therapy

Eleven dogs with severe HA were given AAV8 vectors carrying codon-optimized cFVIII via a dual-chain system (HC + LC, A and E), furin-deleted (ΔF, B and F) or furin-deleted with modified glycosylation sites (ΔF + V3, C and G). One dog was given AAV8 vector carrying cFIX (D and H). Dogs were followed longitudinally for cFVIII activity by chromogenic assay (A–C) or cFIX activity by one-stage assay (D) and cFVIII or cFIX antigen by ELISA (E–H). When unable to obtain appropriate wash-out periods after treatment with FVIII-containing products, a black arrow is included to show the treatment.

thyroxine-binding globulin promoter at 5×10^{13} vg/kg as previously described in two dogs (PC1 and PC2).[16,18] Two HA dogs (PC3 and PC5) received BDD-cFVIII-ΔF, wherein the furin recognition site in the B-domain linker from 1645 to 1648 is deleted (Table S3). This circumvents furin processing and increases secretion of FVIII protein as previously described.[19–21] The remaining severe HA dogs received a novel BDD-cFVIII-ΔF with a modification in which six glycosylation triplets from the B-domain were juxtaposed,[22] referred to as BDD-cFVIII-ΔF + V3 (Table S3). This latter modification in human FVIII (hFVIII) improved packaging of the cassette into AAV as well as hFVIII expression in mice and nonhuman primates.[22] The latter two BDD-cFVIII vectors were under the control of a modified human alpha$_1$-antitrypsin promoter. These transgenes were packaged into AAV8 serotype vectors due to its robust liver tropism to optimize expression. Based upon prior data from research colony dogs, the vector doses for male animals were 3×10^{12} vg/kg for cFIX and 6×10^{12} vg/kg for BDD-cFVIII-ΔF and BDD-cFVIII-ΔF + V3.[8,20] The one female dog (PC10) was dosed at 9×10^{12} vg/kg based upon prior observations that AAV hepatocyte transduction is lower in females.[23]

Longitudinal samples were obtained to monitor cFVIII (chromogenic assay) or cFIX (one-stage assay) activity and antigen by ELISA (Figure 1). The HB dog PC4 had stable cFIX activity of 8.72% and antigen of 7,255 ng/mL at 6 years of follow-up (Figure 1). The median (range) of cFVIII activity in the dogs receiving the dual chain, ΔF, and ΔF + V3 vectors were 1.7% (1.1%–2.4%), 7.2% (2.1%–12.2%), and 4.4% (1.5%–13.8%), respectively (Table 2, and Figure S1A). The cFVIII antigen levels were 7.5 (7.4–7.6), 8.2 (6.1–10.4), and 4.5 (1.0–20.7) ng/mL, respectively (Table 2, and Figure S1B). Although not statistically significant, there was a trend toward higher median cFVIII activity (4.4% vs. 1.7%) in dogs that received gene therapy with the furin-deleted variants compared with the dual-chain system. Given the insensitivity of one-stage assays to detect cFVIII levels below 2%–3%,[6] we tested the one-stage cFVIII activity of dogs with >3% chromogenic cFVIII at time of maximal expression for correlation. As noted in prior studies,[24] one-stage assays yielded higher values than chromogenic assays with an average ratio of 1.7 (range 0.8–3.2) and did not differ by vector type. The cFVIII activity in the companion dogs that received the ΔF vector is comparable to the levels seen in colony dogs that received similar gene therapy.[20] These cFVIII activity levels after gene therapy with furin-deleted cFVIII variants provide comparable activity levels as BDD-cFVIII,[25] but at lower vector doses (Figure S2).

Bleeding events

The types of bleeding events (spontaneous vs. traumatic) and treatments used before and after gene therapy administration are summarized in Tables S1 and S2 and details of location of bleeds following gene therapy in Table S4. These bleeding events were those reported by the caregivers largely due to clinical signs and may underestimate the true bleeding event rate both before and after gene therapy. Nevertheless, the median (range) ABR before gene therapy was 6.7 (2.6–18.9) bleeds/year compared with 0 (0–2.3) bleeds per year ($p < 0.001$, paired t test) following gene therapy (Table 2, and Figure 2A). This represents a 94.1% ± 9.4% (mean ± SD) reduction in bleeding events following gene therapy. Seven of 11 (63.6%) dogs had zero bleeding events following gene therapy (Figure 2B). The percentage of dogs that experienced zero bleeds was higher in those with factor levels >5% (four of five, 90%) compared with those with factor levels of 1%–5% (two of six, 33%). The majority of dogs had provoked bleeding events following gene therapy. PC1 had the most bleeds at 15 bleeding events over approximately 9 years of follow-up, at least

Table 2. Bleeding outcomes and factor expression after AAV gene therapy

Dog	HA/HB	Follow-up (y)	Activity (% normal)	Antigen (ng/mL)	ABR	AIR
PC1	HA	8.88	2.4	7.4	1.7	1.6
PC2	HA	8.49[a]	1.1	7.6	0.5	0.24
PC3	HA	2.56	2.1	10.4	2.3	0.4
PC5	HA	5.55[a]	12.2	6.1	0.4	0.7
PC6	HA	5.03[a]	13.8	12.2	0.0	0.0
PC7	HA	4.34[a]	5.5	2.9	0.0	0.0
PC8	HA	3.41[b]	NA	NA	0.3	0.0
PC9	HA	3.29	3.2	1.0	0.0	0.0
PC10	HA	3.86[a]	12.1	5.3	0.0	0.0
PC11	HA	3.77[a]	1.5	3.8	1.3	1.1
PC12	HA	3.84[a]	2.0	20.7	0.0	0.0
PC4	HB	6.04[a]	8.7	7255	0.0	0.5

ABR: annualized bleeding rate; AIR: annualized infusion rate.
[a]Follow-up ongoing; activity and antigen data from sample at last follow-up as listed.
[b]Lost to follow-up for specimens, follow-up is based upon clinical information provided by owner.

eight of these events were triggered by activities such as hiking or playing with young children in his home setting. In addition, five bleeds occurred in the left leg over a 7-month period consistent with development of a target joint.[26] PC1's bleeds were treated with recombinant cFVIII protein manufactured in-house; other dogs received a combination of fresh frozen plasma, cryoprecipitate, or cFVIII protein (Table S2). The median annualized infusion rate (AIR) was 5.25 (0–15.8) prior to gene therapy compared with 0.12 (0–1.58) after gene therapy ($p < 0.01$, paired t test), consistent with an 88.4% ± 17.9% reduction in infusions per year (Figure 2C). The number of bleeding events (Figure 2B) and infusion events (Figure 2D) trended lower in the dogs who achieved factor levels in the mild (5%–40%) compared with moderate (1–5%) hemophilia range with 0 (0–0.4) vs. 0.9 (0–2.3) bleeds/year and 0 (0–0.7) vs. 0.3 (0–1.6) infusions/year, respectively. The ABR and AIR values at last follow-up did not statistically differ by cFVIII vector type (Figure S3); however, six of seven dogs without bleeding events received the ΔF + V3 vector and the remaining dog was the cFIX recipient. The sole dog who experienced bleeding events after receiving the ΔF + V3 vector had cFVIII expression in the moderate range at 1.5%. Compared with previously published research colony dogs from Queens University,[6] the pre-treatment median ABRs were higher in companion dogs at 6.7 (2.6–18.9) vs. colony dogs at 4.0 (2.1–10.0) bleeds/year ($p = 0.037$ by one-sided t test). After gene therapy, companion dogs with levels in the moderate range (factor levels 1%–5%) had slightly more bleeding events than Queens University and UNC colony dogs with moderate FVIII levels (median 0.9 [0–2.3] vs. 0.1 [0–0.4], data not shown).

Quality of life

To assess impact of gene therapy on QOL, we modified a clinically validated QOL questionnaire developed for dogs with cancer.[27] This survey asks owners the number of days per week their companion dog experienced either positive or negative behaviors and to provide an overall assessment (1–9) of their QOL. These surveys were at last follow-up and owners were asked to retrospectively complete the survey prior to gene therapy concurrently. There was an increase in overall QOL (3.0 ± 2.0 vs. 8.5 ± 0.5, $p < 0.001$ by paired t test). Except for loss of balance and falling, which improved but did not reach statistical significance, all other negative attributes improved compared with pre-gene therapy baseline (Figure 3). Positive physical attributes such as playfulness, ability to participate in daily activities, and acting like themselves improved from baseline but emotional attributes such as enjoying family and being affectionate did not change from baseline (Figure 3). Although there was a trend toward higher scores for enjoying being petted and sleeping through the night, these did not achieve statistical significance.

Safety of AAV gene therapy in companion dogs

Immune responses to cFVIII or cFIX

All dogs tolerated the AAV vector infusion without infusion reactions. All dogs were monitored for inhibitors by Bethesda assay and anti-cFVIII or cFIX IgG2 subclass antibodies (previously demonstrated to correlate with inhibitors[8,28]). Except for PC3 that had a detectable anti-cFVIII inhibitor and IgG2 titer prior to vector administration, there were no detectable inhibitory antibodies to cFVIII or cFIX by Bethesda assay (data not shown) before or after gene therapy and no increase in anti-cFVIII/FIX IgG2 titers from baseline (Figure 4A). Baseline anti-cFVIII or anti-cFIX IgG2 levels were below the average value observed in 50 non-hemophilic dog samples (Figure 4A). Notably, consistent with our prior findings in research colonies,[10] PC3 was able to achieve immune tolerance to cFVIII with decrease in cFVIII inhibitor and IgG2 antibodies (Figure 4B). PC3's pre-existing inhibitor titer was 21 BU/mL and he experienced an anamnestic response after vector infusion with a peak titer of 66.2 BU/mL. This was followed by a gradual decline over the ensuing 2 years to a titer of 1.6 BU/mL. His anti-cFVIII IgG2 subclass antibody followed this same pattern to a negative titer of 1,265 ng/mL at last follow-up. Concurrent with the negative IgG2, he had detectable cFVIII activity and antigen (Figures 1B and 1F).

Hepatotoxicity

Longitudinal liver transaminase levels were available for nine of 12 treated dogs. There were no sustained elevations in aspartate transaminase (AST) or alanine transaminase (ALT) across longitudinal follow-up (Figure S4). PC3, the severe HA dog with pre-existing anti-FVIII inhibitory antibodies, had a baseline elevated ALT at 143 U/L, which remained elevated until 7 months after vector administration. PC2 had a transient increase in ALT at 1.5 years, which resolved without intervention. AST levels were essentially normal throughout the study except for a brief elevation in PC9 at week 5 following vector administration, which also resolved without intervention. There was no evidence of a decrease in FVIII or FIX expression levels during this time. These data are consistent with prior data from colony dogs that the canine model does not recapitulate the capsid-mediated cellular immune response noted in human subjects.

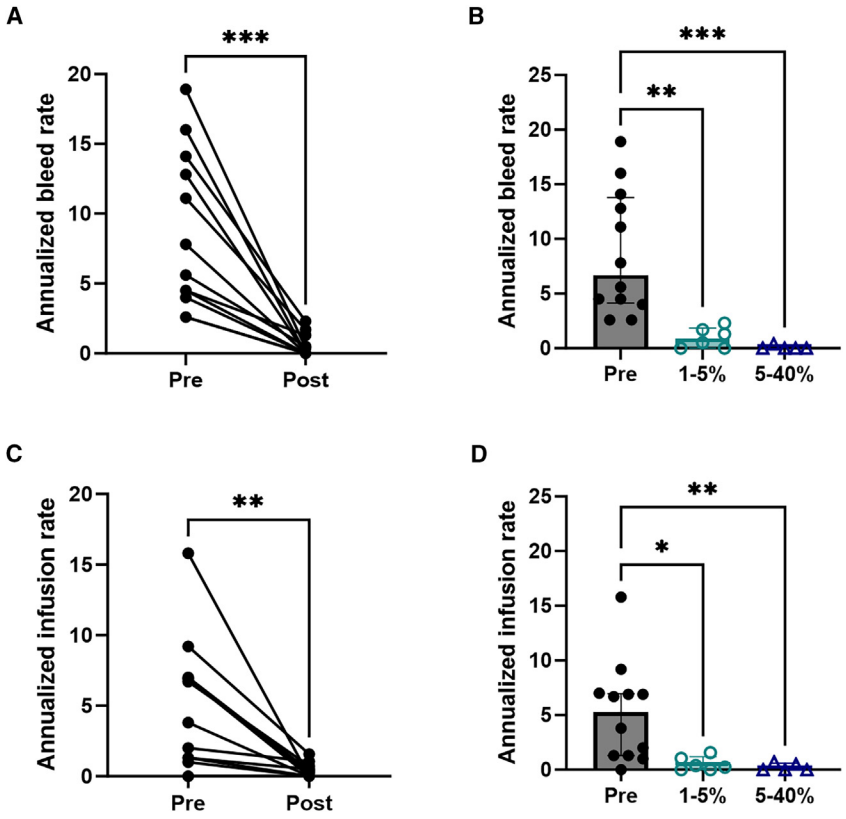

Figure 2. Annualized bleed and infusion rates following gene therapy

Annualized bleed rates are compared before and after gene therapy (A) and by factor level at last follow-up (B). Annualized infusion rates are compared before and after gene therapy (C) and by factor level at last follow-up (D). *p < 0.05, **p < 0.01, ***p < 0.001 by paired t test (A and C) or ANOVA with Dunn's correction for multiple comparisons (B and D). Data shown as median with wrror bars representing interquartile range (B and D).

Serious adverse events

Three deaths occurred during follow-up. PC1, treated with the dual-chain vector experienced extensive trauma-induced hemorrhage in the forelimb, which was associated with neurologic deterioration at approximately 9 years after gene therapy. Due to the acute nature of his deterioration, he was euthanized. Postmortem examination with histopathologic review (brain, heart, lung, spleen, liver, kidney, and left forelimb) confirmed the forelimb bleed but did not inform on the etiology of his neurologic deterioration, as there was no evidence of intracranial hemorrhage or other gross pathology. His last cFVIII activity level prior to this episode was 2.4%. PC3, treated with cFVIII-ΔF vector with a pre-existing anti-cFVIII inhibitor, presented 2 years after gene therapy with acute paraplegia and absent spinal reflexes in hindlimbs. He was intubated due to acute respiratory distress but within an hour progressed to absent corneal and palpebral reflexes and was euthanized. Postmortem examination with histopathologic review (heart, lung, liver, spleen, kidney, thyroid, lymph node, and brain) confirmed severe subdural hemorrhage and severe pulmonary edema with no histologic evidence of infections, metabolic disease, or neoplasia. His cFVIII activity level was 2.1% at time of death. Finally, PC9 treated with the cFVIII-ΔF + V3 vector presented at 3.2 years after gene therapy administration with fulminant and rapidly progressive multicentric lymphoma involving the lymph nodes, spleen, liver, lungs, and mediastinum and was euthanized. This is the most common form of lymphoma in dogs; analysis of the lymphoma samples and normal tissues did not show evidence of vector integration contributing to the development of lymphoma.[18] PC1 and PC9's histology, vector copy number, and genomic integration analysis are reported in Van Gorder et al.[18]; these were not available for PC3. No other dogs have been diagnosed with malignancies in the now 59 years of cumulative follow-up.

DISCUSSION

To our knowledge, our data are the first to describe systemic AAV vector administration for hemophilia in dogs housed in a non-research setting. The 12 companion dogs treated in this study were followed for a median of 4.1 years (range 2.6–8.9) and achieved median cFVIII levels of 2.8% (range 1.1%–13.8%) and antigen levels of 6.7 ng/mL (range 0.9–20.7). Seven of the 11 severe HA dogs had an intron 22 inversion, which is also the most common mutation in severe HA patients; the remainder had previously undescribed *cF8* variants. The severe HB dog achieved cFIX levels of 8.7% and a normal cFIX antigen of 7255 ng/mL. One dog with pre-existing anti-cFVIII inhibitor achieved immune tolerance following gene therapy and no dogs mounted new immune responses to cFVIII or cFIX. There was a ∼94% reduction in ABR and ∼90% reduction in AIR compared with baseline. Together, our data demonstrate that AAV-mediated liver-directed gene therapy is both safe and efficacious in a real-world setting in severe hemophilia A and B companion dogs.

A prior veterinary survey aiming to characterize the clinical presentation and management of hemophilia A in privately owned dogs identified severe hemophilia A (defined therein as <2% FVIII activity) in 16 of 39 (41%) dogs.[29] Of these 16 dogs, 13 (81%) were maintained on periodic blood transfusion support for bleeding events. At a median follow-up of 1.5 years, four of 13 (31%) were euthanized either due to hemophilia A diagnosis or bleeding events despite transfusions given every few months. The annualized bleeding rate seen in our companion dog cohort is similar to this clinical experience at 6.7 bleeds/year. The safety and efficacy parameters reported herein are also similar to those reported in the recent long-term follow-up of research colony dogs housed at Queens University[6] and UNC.[7] The Queens University colony was treated with AAV2, AAV6, and AAV8 serotype vectors via portal vein infusion at 0.6 to 2.7 × 10^{13} vg/kg with follow-up ranging from 8.2–12 years. This study

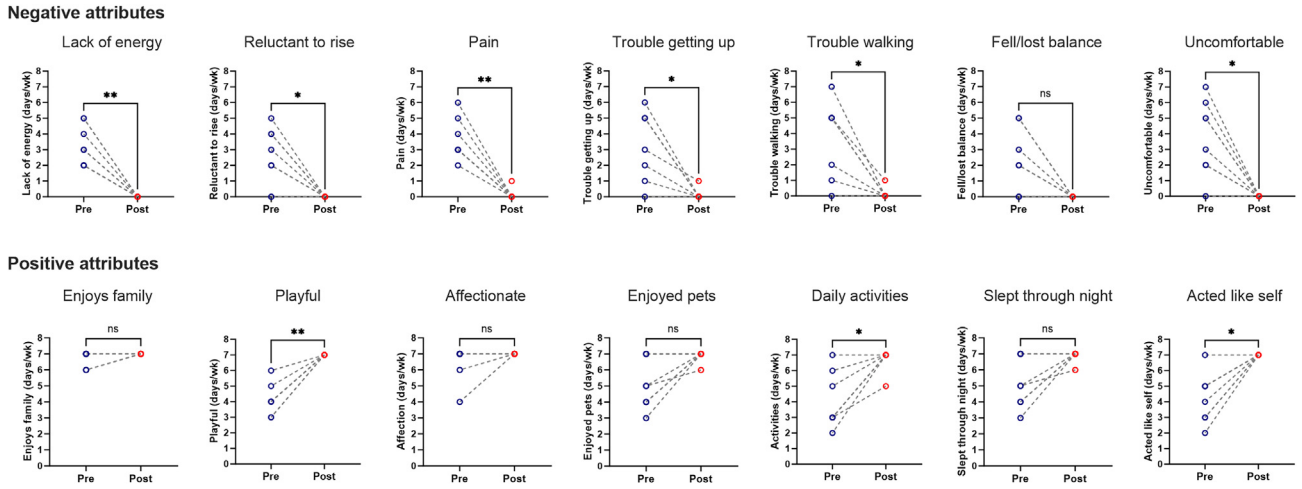

Figure 3. Quality of life before and after AAV gene therapy

Owners were asked to rate the number of days each week that dogs experienced the listed behaviors both before AAV vector administration (pre) and at last follow-up (post). *p < 0.05, **p < 0.01 by Wilcoxon matched signed rank test.

demonstrated persistent cFVIII expression in dogs that responded with a significant decline in ABR from a baseline of 4 to 0.3 events/year. The UNC colony was treated with AAV8 or AAV9 serotype vectors carrying either single- or dual-chain cFVIII at doses of 1.2–4 $\times 10^{13}$ vg/kg. The dogs demonstrated sustained cFVIII expression over follow-up duration ranging from 2.2 to 10.1 years and a post-treatment ABR of 0.13 events/year. The median ABR in companion dogs vs. colony dogs was slightly higher before (6.7 vs. 4.0) gene therapy. The companion dogs reported herein experienced real-world challenges including playing with young children and increased physical activity. Nevertheless, the percent decline in annualized bleeding rate in companion dogs was similar to that seen in the long-term follow-up studies of colony dogs (94% vs. 98%[7] and 92%[6]).

Despite this remarkable success, three severe HA companion dogs died during follow-up. Two of these dogs died from bleeding events with cFVIII expression levels at the lower end of the moderate range (1%–3%) and one developed a target joint. The median ABR in companion dogs vs. colony dogs with factor levels in the 1%–5% range following gene therapy showed a trend toward higher rates in companion dogs (0.9 vs. 0.1, respectively) despite relatively similar cumulative follow-up duration (59 vs. 85 or 60 dog-years, respectively). This increased bleeding signal at lower factor activity levels mimics the human clinical experience where patients with moderate disease do experience bleeding events.[30] Clinical correlation studies in mild and moderate hemophilia A suggest levels >15% are necessary to eliminate joint bleeds.[31–33] Analysis of a phase 3 trial of hemophilia A subjects

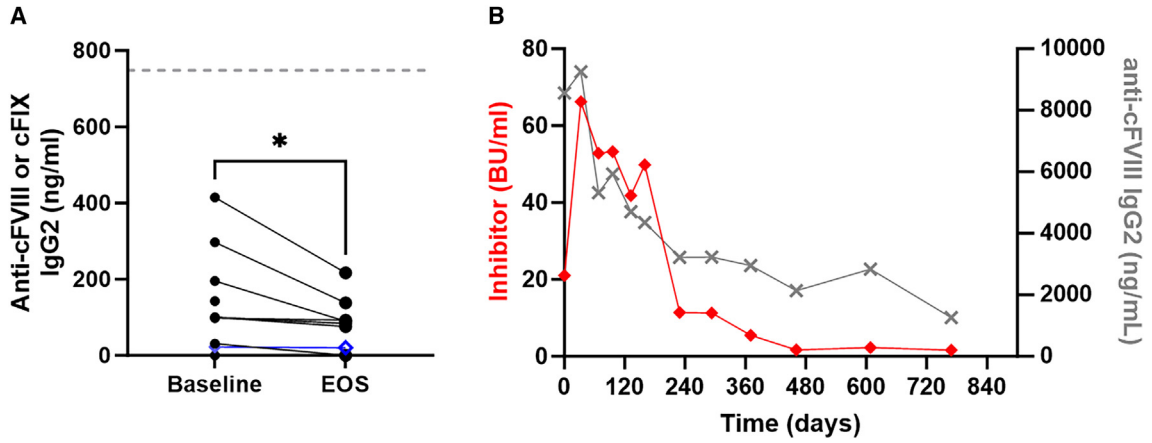

Figure 4. Immune response to cFVIII or cFIX following AAV-liver-directed gene therapy

(A) IgG2 antibodies against cFVIII (●) or cFIX (◇) at baseline and last available follow-up (EOS) for each dog with severe hemophilia except PC3. The gray dotted line indicates mean anti-cFVIII or anti-cFIX IgG2 levels from 50 normal dogs. (B) Inhibitor eradication following gene therapy in PC3 with pre-existing anti-cFVIII inhibitor (red) and IgG2 (gray). *p < 0.05, t test.

also demonstrated an ABR of ∼1 bleed/year with 3%–5% hFVIII expression.[13] Recent guidelines from the World Federation of Hemophilia echo this finding and recommend a goal of FVIII or FIX trough >3%–5% to prevent bleeding complications in the setting of prophylactic clotting factor replacement therapy.[34] Notably, companion dogs with cFVIII and cFIX expression levels >5% had almost no bleeding events and five of six dogs who received the cFVIIIΔF + V3 vector had no bleeds. Together, these data highlight the need for development of alternative strategies (e.g., enhanced transgenes like the cFVIII-ΔF + V3 or FIX-Padua or alternative target tissue) that safely increase transgene expression while minimizing AAV dose-related toxicities. The companion dogs demonstrated stable cFVIII and cFIX activity levels over time, which is consistent with longitudinal results from research colony dogs.[6,7] Although FIX levels have remained steady over multiple years of follow-up in human HB subjects treated with AAV-FIX vectors,[35–38] there has been some loss of expression[14] over time in clinical trials with HA subjects. The reason for the decline seen in human HA subjects is unclear and still undergoing active investigation. Based upon our findings, rapid re-initiation of prophylaxis or close monitoring for bleeding may be warranted in those subjects who lose transgene expression to <5% activity.

Our study is the first to quantify QOL outcomes in hemophilic dogs and demonstrate that the decrease in bleeding and infusion rates after gene therapy correlated with a substantial increase in QOL for companion dogs. Although the retrospective nature of the baseline score may limit the comparison for individual questions, we believe the owners were generally able to give credible overall scores. In the individual domains, the dogs experienced significant improvement in nearly all negative behaviors to less than once or twice per week and showed improvements in physical positive behaviors such as daily activities and playing.

Importantly, there was not an increased signal for anti-cFVIII or cFIX immune responses in companion dogs despite some cFVIII and FFP challenges both for bleeding and surgeries. In fact, we demonstrate the ability to induce tolerance in one companion dog (PC3) with a pre-existing high titer cFVIII inhibitor. This finding supports our prior data from research colony dogs that gene therapy can be used as a one-time infusion that both induces tolerance and provides long-term prophylaxis in the setting of FVIII[10] or FIX[8] inhibitors.[39] The immune tolerance induced by AAV gene therapy in the real-world setting further supports the rationale of studying gene therapy in human HA patients with inhibitors.[40] Notably, tolerance induction in PC3 took nearly 2 years and so patience as well as careful hemostatic management in the interim between vector administration and detectable FVIII activity may be necessary in translational approaches.

Though this study was not designed to evaluate the benefits of AAV gene therapy with furin-deleted cFVIII variants, the sustained disease-ameliorating cFVIII activity levels achieved at vector doses between 3 and 9 $\times$ 10^{12} vg/kg are similar to the sustained cFVIII levels after gene therapy with the standard BDD-cFVIII construct at doses of 0.6–4 $\times$ 10^{13} vg/kg.[6,7] As toxicity of AAV vectors is associated with vector dose,[15] furin-deleted FVIII variants may provide a translatable strategy to enhance HA gene therapy.

In summary, our data support that AAV-liver-directed gene therapy performs comparably in a real-world vs. research setting in a large animal model of hemophilia. Given the increased rates of severe bleeding in dogs with factor expression <5%, our data support a goal sustained factor level >5% to significantly ameliorate the bleeding phenotype and limit morbidity and mortality following gene therapy. Besides the broader translational implications of our study for people with hemophilia, our data combined with recent results from research colony dogs also support the safety and efficacy of AAV gene therapy in companion hemophilic dogs. Our findings suggest that companion dogs could reside in their homes with their owners with decentralized follow-up for factor assays and immunologic screening. Additional studies as well as regulatory and financial hurdles will need to be addressed before AAV gene therapy can enter veterinary clinics.

MATERIALS AND METHODS

Companion dogs

The study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (protocol # 804598) and the Matthew J. Ryan Veterinary Hospital of the University of Pennsylvania (VHUP) Privately Owned Animal Protocol Committee (protocol # 366). Owners or primary care veterinarians became aware of this study by directly contacting researchers at the Children's Hospital of Philadelphia or clinician investigator (M.B.C.) at VHUP. Some dog owners and veterinarians were informed of the study by the Comparative Coagulation Laboratory at Cornell University following a diagnosis of hemophilia A or B. The clinician investigator spoke to all dog owners by phone to determine eligibility, discuss treatment protocol, and arrange additional screening prior to study enrollment. Dogs were enrolled between May 2013 and September 2018. Study inclusion criteria included a severe phenotype (plasma cFVIII or cFIX activity <1% associated with spontaneous bleeding events), age at treatment >6 months, and anti-AAV8 neutralizing antibody titer <1:5. Dogs of any breed and body weight were eligible. Dogs from any state or country were eligible to participate, as long as the owner could travel to VHUP for treatment. All dog owners were required to sign an informed consent form prior to treatment. Clients were responsible for travel costs, but all hospitalization, diagnostic, and treatment fees were covered by the study. The baseline characteristics of these dogs are listed in Table 1 and their bleeding history prior to treatment is listed in Table S1. Therapies to treat bleeding were generally delivered by the local veterinary team except for some dogs who lived in proximity to the Philadelphia region. Clinical follow-up to assess for bleeding was obtained from owners and/or local veterinarians at periodic intervals by the clinician investigator. Periodic plasma samples for factor assays were obtained as convenience samples when the dogs were seen by their local veterinarians.

Molecular diagnosis of hemophilia

DNA was extracted from peripheral blood samples from dogs. For HA dogs, initial PCR sequencing was done to detect the intron 22

inversion mutation as described.[41,42] If negative for intron 22 inversion, 22 pairs of oligonucleotide primers (sequences and PCR conditions available upon request) were used to amplify the coding regions and exon-intron boundaries of the canine *F8* gene for the dogs with hemophilia A. Obtained sequences were aligned and compared with sequences from normal dogs and mammalian species to derive the impact of amino acid substitution on the structure and function of the cFVIII protein as described.[16] For the HB dog, nine pairs of oligonucleotide primers (sequences and PCR conditions available upon request) were used to amplify the exons of canine *F9* gene and sequences obtained were compared with published references sequences.

Canine FVIII and FIX activity, antigen, and antibody assays

Canine FVIII activity was determined by a chromogenic assay (Chromogenix Coatest SP4, Diapharma, Lexington MA) or by one-stage assay as previously described.[6,7] cFVIII antigen levels were measured by ELISA (Affinity Biologicals, Ancaster, ON, Canada) as previously described.[16] Canine FIX activity was measured by one-stage clotting assay cFIX antigen was measured by ELISA (Affinity Biologics, Ancaster, ON, Canada) as previously described.[43] Inhibitory antibodies were measured by Nijmegen modified Bethesda assay.[44] Briefly, plasma samples from companion animals were diluted and mixed 1:1 with normal canine plasma. After a 2-h incubation, the aPTT clotting time was measured in human FVIII or FIX deficient plasma for cFVIII or cFIX antibodies, respectively. IgG2 subclass antibodies to cFVIII or cFIX were determined by ELISA as previously described.[10,43]

Anti-AAV8 neutralizing antibodies

Neutralizing antibodies to AAV8 were determined by an *in vitro* transduction inhibition assay as previously described. Briefly, heat-inactivated serum from dogs was serially diluted and incubated with an AAV8-Renilla luciferase reporter vector for 1 h prior to addition to 2V6.11 cells (ATCC, Manassas, VA)[45] at ~80% confluency in 96-well plates (Millipore Sigma, Burlington MA). The following day, cells were lysed to measure luciferase production (Promega, Madison WI) on GloMax Discover instrument (Promega, Madison WI). The AAV8 titer was determined as the sample dilution that suppressed 50% luciferase expression compared with the negative control.

AAV8 vector production and administration

Recombinant AAV vectors were produced by triple transfection protocol as previously described, using plasmids expressing (1) wild-type canine FIX or BDD canine FVIII, which retains a 14-amino acid linker, (2) plasmid supplying adenovirus helper functions, and (3) plasmid containing the AAV2 *rep* and the AAV8 *cap* genes. Vectors were purified by cesium chloride density gradient centrifugation and titers obtained via Taqman PCR (Applied Biosystems, Förster City CA).[10] Vector doses are listed in Table 1.

The vectors were prepared in sterile PBS in a total volume of 10 mL/kg body weight and injected via the saphenous vein. All dogs received 50 IU/kg of recombinant purified cFVIII protein or FFP for HB dog 30 min prior to AAV vector administration. Dogs were monitored in the hospital for 2 days following vector administration.

Recombinant protein

Canine BDD-FVIII was expressed from mammalian cells and purified to homogeneity as previously described.[8–10,20] A dose of 50 units/kg was given to each severe HA dog prior to vector infusion as well as with bleeding events for those that were within our local region. Recombinant canine factor Xa variant I16L was expressed and purified as previously described for human FXa I16L (manuscript in preparation).[46]

DATA AND CODE AVAILABILITY

All original data are available from the authors on request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2024.101205.

ACKNOWLEDGMENTS

The authors would like to sincerely thank the owners of these companion dogs for entrusting their pets to this research study and the primary care veterinarians who helped with case management and sample collection. We also acknowledge research funding from NHLBI (HL158781 to V.R.A. and B.J.S.J., HL156073 to B.S.D., and N01_75N92019D00041 to T.C.N.), the National Center for Advancing Translational Sciences (UL1TR000003 to V.R.A. and M.B.C.) and the Institute for Translational Medicine and Therapeutics of the Perelman School of Medicine and the School of Veterinary Medicine at the University of Pennsylvania (V.R.A. and M.B.C.). We thank Rodney Camire for provision of factor Xa I16L protein and Denise Sabatino for providing bleeding data on colony dogs. Finally, we thank the Children's Hospital of Philadelphia's Research Vector Core for their help in producing the AAV vectors.

AUTHOR CONTRIBUTIONS

B.S.D. designed and performed experiments, analyzed data, and wrote the manuscript. B.J.S.J., E.P.M., R.A.F., J.L.S., and B.J.L. designed and performed experiments, analyzed data, and revised the manuscript. T.C.N., V.R.A., and M.B.C. supervised experiments, analyzed data, and revised the manuscript. V.R.A. designed and supervised experiments but passed away prior to completion of the manuscript. M.B.C. provided clinical care to the animals as part of the research protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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