



Adeno-associated viral vector gene therapy for canine hemophilia

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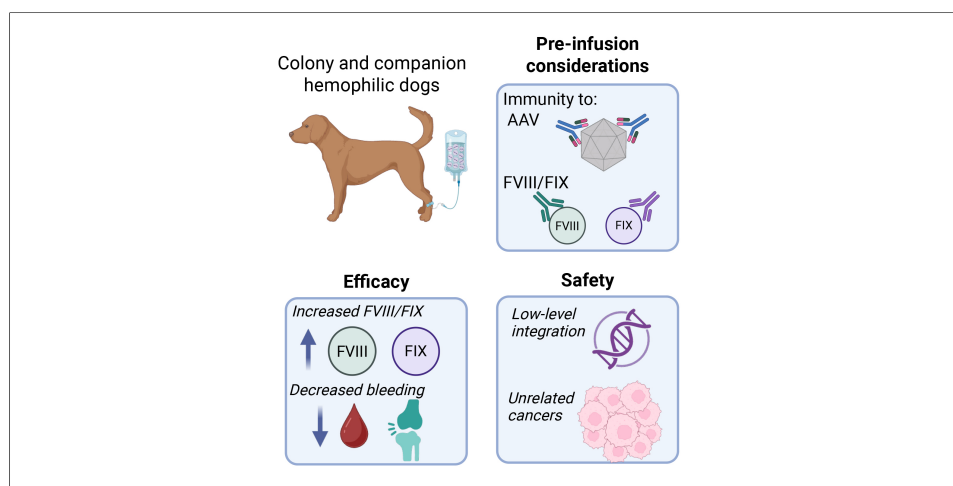
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Abstract

Hemophilia A (HA) and B (HB) are monogenic X-linked disorders caused by plasma coagulant factor VIII (FVIII) and IX (FIX) deficiency, respectively, and occur naturally in a variety of dog breeds. While several animal models of hemophilia exist, the canine model closely mimics the genotypic, phenotypic, and immunologic characteristics of the disorder in humans. Consequently, decades of research were conducted in the canine model of hemophilia, ultimately leading to licensed liver-directed adeno-associated viral (AAV) vector-mediated gene therapy for HA and HB in humans. The studies from both research colony and companion hemophilic dogs with AAV liver-directed gene therapy support the safety and efficacy of this platform in canines. Here, we provide practical considerations with respect to the administration and monitoring of liver-directed AAV gene therapy for hemophilia in veterinary clinical practice and review the data on efficacy and safety of AAV gene therapy in canine hemophilia.



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INTRODUCTION

Hemophilia is an inherited bleeding disorder that occurs in humans and naturally in many animal species, including dogs, cats, cattle, horses, and sheep. Hemophilia A (HA) and B (HB) are caused by plasma coagulant factor VIII (FVIII) and IX (FIX) deficiency, respectively. Hemophilia is an X-linked recessive trait affecting primarily males, although females can experience bleeding with low coagulation factor levels resulting from skewed X chromosome inactivation in heterozygous female carriers, or, rarely, homozygous inheritance^[1]. While there are several animal models of hemophilia, the genotypic, phenotypic, and immunologic characteristics of the naturally occurring canine model most closely mimic the disorder in humans^[2-6]. Decades of research using the canine model of hemophilia ultimately led to clinical trials evaluating the safety and efficacy of liver-directed adeno-associated virus (AAV) vector-mediated gene therapy for HA and HB in humans, and canine models contribute to ongoing gene therapy development in other monogenic disorders^[7]. In 2022, the US Food and Drug Administration (FDA) approved etranacogene dezaparvovec-drlb (Hemgenix®) as the first one-time gene therapy for adults with HB^[8], followed by approval of valoctocogene roxaparvovec-rvox (Roctavian®) in 2023 for HA. Given that AAV gene therapy has proven to be a safe and effective treatment for research dogs with hemophilia, this novel therapy was offered to a limited number of companion dogs with HA and HB as part of an investigational study, with a 94% reduction in bleeding rates and an improved quality of life (QOL)^[9,10]. There is a continued and growing interest in making gene therapy available to more companion dogs with hemophilia that exhibit a severe phenotype.

HEMOPHILIA IN DOGS

Clinical presentation and epidemiology

The bleeding phenotypes for both dogs and humans with hemophilia correlate with the degree of plasma coagulant factor deficiency. Both HA and HB are characterized as severe when factor activity is < 1% of normal, moderate for factor activity between 1% and 5%, and mild for factor activity between 5%-40%^[11]. Dogs and humans with severe hemophilia exhibit frequent spontaneous bleeds, most commonly hematoma formation (in muscle or subcutaneous tissue), hemarthrosis, and oral bleeding associated with tooth loss or eruption, though other mucosal surface bleeding (e.g., epistaxis, gastrointestinal bleeding, hematuria) and hemorrhage into body cavities (thoracic pleural space, mediastinum, peritoneum, retroperitoneum) and the central nervous system can occur. While bleeding phenotypes can be variable in moderate and mild disease, moderate hemophilia typically results in traumatic or post-surgical bleeding with rarer spontaneous bleeding while those with mild deficiency generally have bleeding induced by surgery or trauma.

HA is far more common than HB in both dogs and humans. The mean estimated prevalence of HA and HB in humans is reported globally as 17.1 and 3.8 per 100,000 males, respectively^[12]. There are insufficient data to determine the prevalence of hemophilia in dogs. Between 2018 and 2025, the Comparative Coagulation Laboratory at Cornell University diagnosed an average of 97 dogs per year with HA (~8-10 cases per month) and 8 dogs per year with HB (personal communication with Dr. Marjory Brooks, Comparative Coagulation Laboratory, Cornell University). Given that dogs with severe hemophilia might experience fatal hemorrhage or be euthanized before a diagnosis is confirmed and diagnostic evaluation might not be pursued for dogs with mild hemophilia due to infrequent bleeding events, the prevalence of canine hemophilia is likely under-recognized. Indeed, a prior veterinary study of FVIII-deficient dogs identified severe HA in 16 of 39 (41%) animals, four of whom were euthanized either because of their hemophilia diagnosis or due to severe bleeding despite transfusions^[13].

Diagnosis

For dogs that exhibit a bleeding tendency, such as repeated spontaneous hemarthrosis or hematoma formation or excessive bleeding after surgery (e.g., scrotal hemorrhage post-castration) or trauma, initial

diagnostic evaluation typically includes measurement of a platelet count, prothrombin time (PT), and activated partial thromboplastin time (aPTT). Dogs with HA or HB have a prolonged aPTT and normal PT, indicating an intrinsic coagulopathy. In a male dog, the most likely explanation for prolonged aPTT only is hemophilia. Plasma coagulation factor analysis is necessary to identify the specific factor deficiency. In the US, the Comparative Coagulation Laboratory at Cornell University is the reference laboratory for coagulation factor analysis. The required sample is citrate plasma. For accurate results, pre-transfusion blood samples should be obtained via clean venipuncture directly into sodium citrate tubes, with a citrate: blood ratio of 1:9. Factor VIII and FIX coagulant activities are measured using pooled canine FVIII- and FIX-deficient substrate plasma, respectively, in a modified aPTT assay. Results are reported as percentage FVIII and FIX coagulant activity compared to pooled normal canine plasma, which has an assigned value of 100% (reference interval, 50%-150%).

Although measurement of plasma FVIII and FIX coagulant activity is accurate for the diagnosis of hemophilia in dogs with a bleeding tendency, it does not always distinguish between carrier and clear females^[14]. DNA testing can be performed for breeds with known disease-causing mutations to guide breeders with their mating decisions in an effort to eliminate the trait from their line^[14-16]. The *F8* and *F9* genes are localized to the X chromosome in dogs and humans. The *F8* gene is much larger than the *F9* gene in both species, consisting of 26 and 8 exons, respectively, with the mature FVIII and FIX proteins having 2,332 (2,343 in the dog) and 415 (452 in the dog) amino acids, respectively. More than 4,000 *F8* and 1,400 *F9* variants have been identified in humans with HA and HB, respectively, and compiled in various global databases. In a genotyping study of more than 11,000 humans with hemophilia, *F8* and *F9* variants predicted to be gene-disrupting were common in severe hemophilia, while missense variants predominated in moderate and mild hemophilia^[17]. Several disease-causing mutations have been identified in dogs with HA and HB [Table 1]. An intron 22 inversion of the *F8* gene is the most common *F8* mutation in both humans and dogs with severe HA^[10].

DNA testing for canine hemophilia is available for specific breeds through the Veterinary Genetics Laboratory at the University of California-Davis and the Comparative Coagulation Laboratory at Cornell University, as well as commercial dog DNA testing companies (e.g., Embark, Orivet, Wisdom Panel). Given that hemophilia is an X-linked recessive trait, there are 2 possible genotypes for male dogs: clear (normal *F8* or *F9* gene on X chromosome) or affected (*F8* or *F9* variant causing hemophilia on X chromosome). For females, there are 3 possible genotypes: clear (normal *F8* or *F9* gene on both X chromosomes), carrier (normal *F8* or *F9* gene on one X chromosome and *F8* or *F9* variant on the second X chromosome), or affected (*F8* or *F9* variant on both X chromosomes). Affected male and female dogs are typically evident based on their bleeding phenotype and plasma coagulant factor activity, but DNA testing is necessary for accurate detection of carrier females. Breeding a carrier female to a clear male will result in approximately 50% of the female offspring being carriers and 50% of the male offspring being affected with hemophilia. Therefore, carrier females should not be used for breeding.

Current standard of care in veterinary practice

Factor replacement therapy

The current standard of care for humans with severe HA and HB is prophylactic administration of recombinant human FVIII and FIX concentrates, respectively^[31]. Patient compliance, poor venous access, and development of inhibitors are potential issues with prophylactic treatment with factor concentrates. Recombinant human FVIII and FIX concentrates have been administered to research dogs with HA and HB, respectively, and were efficacious in improving the results of hemostatic tests, but treated dogs developed inhibitory anti-human FVIII and FIX antibodies within 14 days^[32,33]. While recombinant canine FVIII and FIX concentrates have been available in a research setting^[10,34], there are no commercially available canine

Table 1. Genetic variant identified in dogs with hemophilia

Gene	Variant type	Variant	Breeds affected	References
F8	Intron 22 inversion	Intron 22 inversion	<i>Research colonies:</i> Irish Setter, Miniature Schnauzer <i>Companion dogs:</i> Staffordshire terrier, French Bulldog, Labradoodle, Cocker Spaniel, Shih Tzu, mixed breed	[10,18,19]
F8	Missense	Thr62Met	Shepherd mix	[10]
F8	Small deletion	Exon 14 10-bp deletion	Mixed breed	[10]
F8	Complex*	Complex*	Golden Retriever	[10]
F8	Small deletion	Exon 14 2-bp deletion	Labrador Retriever	[20]
F8	Nonsense	Exon 1 point mutation (TGG>TAG), Trp33Stop	German Shepherd	[21]
F8	Nonsense	Exon 12 point mutation (CGA>TGA), Arg577Stop	Old English Sheepdog	[22]
F8	Missense	Pro471Arg	Boxer	[16]
F8	Missense	Cys548Tyr	German Shepherd	[16]
F8	Small deletion	Exon 14 1-bp deletion p.Asn1069IlefsTer7	Border Collie	[15]
F8	SINE insertion	Exon 14 SINE insertion	Rhodesian Ridgeback	[23]
F8	SINE insertion	Exon 14 SINE insertion (220 bp)	Havanese	[21]
F9	Missense	Glu379Gly	<i>Research colony:</i> Cairn Terrier	[24]
F9	Small deletion/transition	5-bp deletion and transition p.Arg183Leufs*3	<i>Research colony:</i> Lhasa Apso	[25]
F9	Large deletion	Deletion of the entire gene	Labrador Retriever	[26]
F9	Large deletion	Deletion 5' to exon 6	American Pit Bull Terrier	[27]
F9	Insertion	Exon 8 5-kb insertion	Airedale Terrier	[27]
F9	Insertion	Intron 5 1.5-kb insertion	German Wirehaired Pointer	[14]
F9	Missense	Gly244Glu	Rhodesian Ridgeback	[28]
F9	Small deletion	Single nucleotide deletion in the promoter	Hovawart	[29]
F9	Insertion	Exon 8 insertion p.Asn274LysfsTer23	Newfoundland	[30]
F9	Missense	Gly12Arg; Ala28Thr	Boston terrier	[10]

*ex18 insertion poly (A) tract, 14nt duplication, p.Y1987Stop.

factor concentrates for use in clinical veterinary practice. Therefore, blood component therapy is the mainstay of managing severe bleeding. Furthermore, primarily due to expense, dogs with hemophilia are treated at the time of bleeding events rather than as a weekly prophylaxis. For dogs undergoing surgery, prophylactic protein replacement therapy is recommended immediately prior to surgery and then ideally every 8–12 h postoperatively for 1–2 days to provide adequate hemostasis. In general, it is more efficient to prevent bleeding than to react to bleeding postoperatively.

Canine blood products that contain functional coagulation factors include fresh whole blood (FWB), fresh-frozen plasma (FFP), cryoprecipitate (CRYO), and cryosupernatant or cryo-poor plasma (CPP). To maximize the use of each unit of FWB and minimize the risk of some transfusion-associated adverse events, ideally, the blood product selected should treat the specific need of the patient, i.e., replacement of plasma FVIII or FIX for HA or HB, respectively. FFP contains all coagulation factors and can be used to manage bleeding in dogs with HA or HB; therefore, it would be an appropriate choice for a male dog with a prolonged aPTT and presumptive diagnosis of hemophilia while awaiting results of coagulation factor analysis to determine if the dog has HA or HB. CRYO is prepared from FFP and is a concentrated product containing FVIII, von Willebrand factor (VWF), fibrinogen, fibronectin, and FXIII; it is the blood component of choice for management of bleeding in dogs with HA^[35]. CPP contains FIX and other

coagulation factors that are not present in CRYO, making it an excellent choice for management of bleeding in dogs with HB. If CRYO or CPP is not available, FFP is an appropriate choice for dogs with HA or HB. In addition, a red blood cell (RBC) transfusion (FWB or packed RBCs) might be necessary if an affected dog becomes moderately to severely anemic due to extensive bleeding.

The most serious complication of exogenous coagulation FVIII or FIX replacement is the development of inhibitors [immunoglobulin G (IgG) alloantibodies] that neutralize the function of infused factor replacement therapies. In both humans and dogs with hemophilia, the presence of an inhibitor is suspected when previously treated patients subsequently fail to respond clinically to protein replacement therapy. Inhibitors are measured by the Bethesda assay, with a positive inhibitor defined as a Bethesda titer of > 0.6 Bethesda units (BU)^[11]. In humans, a low-responding inhibitor (defined as < 5.0 BU) tends to be transient and drop below threshold within 6 months, whereas a high-responding inhibitor (≥ 5.0 BU) tends to persist but may become undetectable after a long period without protein replacement therapy; however, inhibitor titers will increase 3–5 days after rechallenge with protein replacement^[11].

In humans, inhibitors are more common in HA than HB and in severe hemophilia compared to mild to moderate disease. The reported incidence of inhibitors in humans with severe HA and HB is 30% and 12%, respectively, and with mild/moderate HA and HB 9% and 2%, respectively^[17]. Other risk factors for the development of inhibitors in humans include high-impact (i.e., gene-disrupting) *F8* or *F9* variants, the type and intensity of protein replacement therapy, and, for HA, race/ethnicity^[36–38]. Most of the information available regarding inhibitors in dogs is based on data from the HA dog colonies at the University of North Carolina at Chapel Hill (UNC-CH) and Queens University (QU) and the HB dog colonies at the University of Alabama (UAB) and UNC-CH^[18,19,24,25]. Dogs with HA at both UNC-CH and QU have the intron 22 inversion *F8* variant, yet the QU dogs, all from a common female ancestor, are considered “inhibitor-prone”, with approximately 25% developing inhibitors after protein replacement therapy. The UNC-CH dogs generally have a lower rate of inhibitor formation, although a separately bred subset of the colony is inhibitor-prone, indicating that genetic factors other than the disease-causing mutation impact the risk of inhibitor development^[18,39,40]. The HB dogs at UAB have a *F9* deletion variant and are “inhibitor-prone”, whereas the UNC-CH HB dogs have a *F9* missense mutation and tend not to develop FIX inhibitors^[39].

The incidence of inhibitors among companion dogs is largely unknown. Twelve dogs, 11 HA and 1 HB, were screened for inhibitors before adeno-associated viral (AAV) type 8 (AAV8) gene therapy^[10]. Seven of 11 dogs had the intron 22 inversion mutation, and only 1 of these 7 dogs had a FVIII inhibitor; all other dogs tested negative^[10]. Among other HA and HB dogs that have been genotyped, inhibitor screening was reported for only 2 dogs. An Old English Sheepdog with HA due to a nonsense mutation tested negative for FVIII inhibitors despite extensive treatment with FVIII to manage 55 bleeding events^[22]. However, a Labrador Retriever with HB due to deletion of the entire FIX gene developed a low-responding inhibitor (3 BU) after a second plasma transfusion and a high-responding inhibitor (14.5 BU) after a third high-dosage plasma transfusion^[26].

Alternatives to factor replacement therapy

In clinical veterinary practice there are currently no alternatives to blood component therapy for management of bleeds in dogs with hemophilia. For human patients experiencing breakthrough bleeds despite prophylaxis, factor concentrates are recommended for those with no or low-titer inhibitors, while bypass agent therapy, either recombinant human FVIIa (rhFVIIa) or activated prothrombin complex concentrate (aPCC), is recommended for those with high-titer inhibitors^[31]. Administration of rhFVIIa improved results of hemostatic tests in research dogs with HA (both with and without FVIII inhibitors) and

HB, but the dogs developed anti-human FVIIa antibodies within approximately 2 weeks^[41,42]. In a single research dog with HA, administration of recombinant canine FVIIa (rcFVIIa) was well tolerated and corrected the hemophilic coagulopathy^[43]. Unfortunately, rcFVIIa is not commercially available. Therefore, for companion dogs that have developed FVIII or FIX inhibitors, supraphysiologic amounts of coagulation factor must be administered to overcome the inhibitors and provide hemostasis, with the negative consequence of further inhibitor formation.

Antifibrinolytics

Bleeding in hemophilia results from impaired clot formation, as well as premature lysis of the clot resulting from reduced clot stability due to the lack of amplified and persistent thrombin generation^[44]. Administration of tranexamic acid (TXA), an antifibrinolytic that competitively inhibits the conversion of plasminogen to plasmin, combined with rhFVIII, increased clot resistance to accelerated fibrinolysis in humans with severe HA compared with factor replacement alone^[45]. Similarly, in research dogs with HA, clot stability measured using thromboelastography in plasma samples collected at baseline and 4 h after intravenous administration of TXA (10 mg/kg) demonstrated delayed clot lysis at 4 h^[46]. For humans with hemophilia, the World Federation of Hemophilia recommends antifibrinolytics as an alternative to use alone or as adjuvant treatment, particularly in controlling superficial soft tissue and mucosal bleeds (e.g., epistaxis, oral and gastrointestinal bleeding, and menorrhagia) and for dental surgery and eruption or shedding of teeth^[31]. Similarly, minor bleeding events in dogs with hemophilia were controlled with TXA administered at 15–25 mg/kg orally every 6–8 h for approximately 5–7 days. Aminocaproic acid is an alternative antifibrinolytic, although it is used less widely in humans due to its shorter plasma half-life, lower potency, and higher toxicity compared to TXA^[31]. For dogs with moderate to severe bleeding events that require protein replacement therapy, an antifibrinolytic can be administered as an adjunctive treatment.

Desmopressin

Desmopressin (1-deamino-8-D-arginine vasopressin, DDAVP) is a vasopressin type 2-receptor (V2R) agonist that causes endothelial cells to secrete stored VWF and FVIII into the bloodstream. Bleeding in humans with mild to moderate HA can be treated or prevented with DDAVP, which typically results in a 3- to 4-fold increase in basal FVIII levels in this patient population^[47]. However, the response to DDAVP varies widely between individuals, with basal FVIII levels and the *F8* gene defect influencing the response^[47]. In contrast, the DDAVP-induced FVIII response in dogs is less robust, with normal dogs having a 1.3–1.5-fold increase in FVIII activity over basal levels^[48,49]. There are limited data on efficacy of DDAVP in dogs with HA. Four dogs with mild HA (FVIII 10%–24%) treated with DDAVP (0.4–5 µg/kg SC) had a negligible increase in FVIII activity^[50]. Not surprisingly, DDAVP administration (5 µg/kg IV) had no effect on FVIII activity in two research dogs with severe HA that had received retroviral vector gene therapy^[49]. Desmopressin does not appear to be an effective adjunctive treatment in managing bleeding in dogs with HA, regardless of severity of disease.

Limitations compared to gene therapy

Factor replacement therapy, the mainstay of management of bleeding events in dogs with hemophilia, has a transient benefit. For dogs with severe HA or HB, some might require monthly transfusions to control spontaneous bleeds, resulting in a large financial burden for the client over time and, therefore, potentially euthanasia^[13]. In contrast, AAV gene therapy for HA and HB provides a one-time treatment that yields a sustained increase in plasma FVIII or FIX activity for years (> 10 years documented in some research and companion dogs), a marked (94%) reduction in bleeding events, and an improved QOL^[10].

LIVER-DIRECTED AAV GENE REPLACEMENT THERAPY IN CANINE HEMOPHILIA

As canine hemophilia closely mimics the human disease^[2-6], studies in hemophilic dogs were informative in the development of HA/HB gene therapies for humans. Initial *in vivo* studies using retroviral or adenoviral vectors carrying either human or canine *F8* and *F9* complementary DNA (cDNA) were unsuccessful due to transgene silencing or the development of immune responses directed against either the vector, transduced cells, or the secreted xenoprotein that could not be rescued with immunosuppression^[51-55]. Given these limitations, alternative vectors were investigated. Successful preclinical AAV vector gene therapy in other disease models^[56,57] provided evidence to study AAV vectors in hemophilia. AAVs are single-stranded, nonpathogenic, replication-defective DNA viruses belonging to the Parvoviridae family^[58]. The AAV genome is ~4.7 kb and comprises inverted terminal repeats (ITRs) surrounding replication and capsid genes; in vector manufacturing, these genes are replaced with the transgene and promoter of interest^[59]. The capsid serotype provides some tissue specificity, which can be further refined by the promoter to limit transgene expression to the tissue of interest^[60].

AAV vectors were initially studied in HB^[61-63] owing to the smaller cDNA size of *F9* (1.6 kb)^[64] vs. full-length *F8* (7 kb)^[65,66] which could not be packaged into the AAV capsid. Identification of B-domain deleted (BDD) FVIII, which removed ~2.6 kb, in combination with other strategies, permitted investigation of these shortened *F8* transgenes in AAV applications^[67-70]. The ensuing decades of preclinical work^[5,71,72] in animal models of hemophilia informed the development, and eventual licensure, of AAV vectors for HA and HB in people^[8,73-77]. These preclinical canine studies were completed in dogs housed in research colonies^[72,78-93]. A clinical study in companion dogs was also completed, informing on real-world safety and efficacy of AAV gene therapy in dogs with hemophilia^[9,10]. Here, we provide practical considerations with respect to the administration and monitoring of liver-directed AAV gene therapy for hemophilia in veterinary clinical practice and review the data on efficacy and safety of AAV gene therapy in canine hemophilia that led to the clinical development and approval of AAV gene therapy for human hemophilia.

Pre-infusion and eligibility considerations

AAV-based gene therapy is largely indicated for dogs with severe disease (< 1% FVIII or FIX) to ameliorate the severe, recurrent bleeding events. While there is a mutational spectrum in canine hemophilia [Table 1], the outcomes after gene therapy do not appear to be impacted by the underlying genetic defect^[9,10,18,19,22,72,82,90,93,94]. Therefore, functional activity assays to identify dogs with severe hemophilia have been sufficient to identify dogs that would be eligible to receive AAV vectors. However, proclivity for anti-FVIII or FIX inhibitors is associated with more severe genetic defects that result in no antigen production. Thus, testing for the presence of inhibitors prior to vector infusion for dogs exposed to replacement therapies and blood products is an important consideration.

Several studies in research colony-housed HA dogs have shown that liver-directed AAV gene therapy can both promote immune tolerance and provide sustained therapeutic FVIII levels. These studies have been completed in two primary canine HA research colonies (described above) with intron 22 inversion of *F8*^[18,19]. The QU colony has historically been inhibitor-prone compared to the initially immune-tolerant colony at UNC-CH, though outbreeding of the UNC-CH colony has resulted in development of inhibitor-prone dogs there as well. More recently, the colony at UNC-CH has included a high responding inhibitor dog colony with a large gene deletion and emerging data from AAV gene therapy from this model suggests that AAV-mediated immune tolerance may be mutation and peak inhibitor titer-dependent^[95]. Thus, the severity of the *F8* mutation and the pre-AAV infusion anti-FVIII inhibitor titer may be important in predicting response to AAV gene therapy in the setting of an anti-FVIII immune response; this has also been borne in human disease^[96-99]. In contrast to people with HB with FIX inhibitors, dogs with FIX inhibitors do not develop allergic symptoms or nephrotic syndrome^[100]. Notably, HB dogs in research colonies with inhibitors

have demonstrated the ability to induce tolerance to FIX, in addition to achieving therapeutic FIX levels, following AAV gene therapy with a hyperactive FIX variant (FIX-Padua)^[72,93].

Another consideration for eligibility is the presence of anti-AAV antibodies at the time of infusion. Titers as low as 1:5 can ablate liver transduction following systemic delivery of AAV in mice^[101], and a human patient with a 1:17 titer had a dramatic reduction in the efficacy of his gene therapy^[102]. Yet, despite the high seroprevalence of anti-AAV antibodies in the human population^[103], the same has not been seen in canines. The authors of this paper have worked with eight canine colonies covering four disease models and wild-type animals and have never had to exclude a dog for pre-existing anti-AAV antibodies. One of the large colonies in question at UNC-CH, run by Dr. Timothy Nichols, has only had one experiment impacted by anti-AAV antibodies (personal communication) despite decades of experience using AAV in dogs. In that instance, two dogs likely developed anti-AAV antibodies from exposure to treated animals shedding the vector (see section below on vector shedding)^[104]. Notably, in our work to treat companion dogs with hemophilia with AAV, we have not had to exclude any animals. All twelve of the companion dogs we have treated had anti-AAV titers < 1:5 at the time of infusion^[10]. Importantly, this is despite routine vaccination protocols in both the dog colony and companion animals that include the canine parvovirus vaccine.

Infusion and monitoring after AAV gene therapy

While portal vein injection was used in early canine studies, more recent studies deliver the AAV vectors via peripheral intravenous infusion, often using the cephalic or saphenous vein, in a sterile saline solution^[10,94,105]. In both delivery methods, research colony and companion dogs received hemostatic therapy (either recombinant purified canine FVIII protein or FFP) immediately prior to AAV vector administration. Companion dogs were admitted and monitored in the hospital for 2 days following vector administration^[9,10].

A short-term toxicity consideration in human AAV liver-directed gene therapy for hemophilia is the occurrence of a cellular immune response directed against the vector capsid. This capsid-directed response was not observed in preclinical murine and non-human primate AAV studies. However, it was noted in an early AAV type 2 (AAV2)-FIX gene therapy trial, in which approximately 4 weeks after vector infusion, a patient developed an asymptomatic rise in alanine aminotransferase (ALT) that coincided with a gradual loss of FIX expression over the ensuing 8–12 weeks^[102]. Using an ELISPOT assay, that study showed evidence of activated T cells against the vector capsid, but not against FIX, confirming the hypothesis that transduced liver cells presenting the capsid were targeted for destruction by the host immune system. A follow-up study showed that corticosteroids could dampen the cellular immune response when a rise in ALT was observed and rescue FIX expression^[73].

This capsid-directed cellular immune response has not borne out in canine hemophilia gene therapy studies with long-term follow-up^[10,94,105]. In the UNC-CH colony dogs, ALT levels were measured two to four times per year and remained normal in 6/9 dogs at most of the timepoints analyzed. Two dogs who were older at time of vector infusion had minor ALT elevations (< 2 times upper limit of normal) starting 4 years after AAV administration and one dog had ALT elevations > 2 times the upper limit. However, there was no specific liver disease pattern (AST levels were within normal limits for all dogs) and serum α -fetoprotein (AFP) was normal at all timepoints. Postmortem examinations of the liver were conducted in both studies and compared to those of untreated age-matched dogs. In the QU research colony dogs, post-mortem examinations did not reveal any significant parenchymal inflammation, necrosis, hepatocyte injury, fibrosis, adenoma, or carcinoma^[94]. In the UNC-CH colony dogs, there were no AAV-treated liver pathologic changes observed compared to untreated dogs^[105]. In the companion dog study, there were no sustained elevations in AST or ALT across longitudinal follow-up and there was no evidence of decreased FVIII or FIX expression at times of transient elevations^[10]. Together, these data suggest that hemophilic dogs do not

experience the capsid-mediated cellular immune response noted in human subjects.

Unfortunately, no animal models faithfully recapitulate the anti-capsid cellular immune response seen in humans. While there are numerous hypotheses as to why this might be - including inbred strains having restricted major histocompatibility complex (MHC) haplotype pools^[106], humans having previous exposures to wildtype AAVs^[107], and humans having hyperactive T cells due to a CMP-N-acetylneuraminic acid hydroxylase (CMAH) mutation^[108] - none have borne out. Outbred animal models including dogs and primates with broad MHC haplotypes do not mount such responses; previous vaccination against AAV still does not induce anti-capsid T cells in mice^[107], and CMAH^{-/-} mice do not have destruction of AAV-transduced hepatocytes^[108].

Vector shedding guidance

In humans, AAV shedding in the urine is largely complete by one week after treatment^[102]. Note that this “shedding” refers to detectable DNA sequences, not necessarily intact vector or vector that retains the ability to transduce. While it can persist longer in semen, in rabbits AAV shed in semen is no longer able to transduce by day 7^[109], and in mice the shed vector may never be capable of transduction^[110]. Nonetheless, in a recent study intending to treat HB dogs with intravenous AAV at 2 days, 30 days, 3 months, and 6 months of age, the animals treated at 30 days of life did not express detectable levels of FIX^[104]. Interestingly, these dogs had frequent interactions with dogs treated at 2 days of life over the subsequent 28 days, a period during which the latter would be expected to shed vector (personal communication, Timothy Nichols, University of North Carolina at Chapel Hill, Chapel Hill, NC, USA). Both dogs treated at 30 days were subsequently found to have anti-AAV antibodies. Due to the theoretical risks of vector shedding and the potential for acute adverse events, we have previously admitted companion animals for the 48 h after vector administration. However, given the lack of realized adverse events in human trials, the current plan is for future dogs to be treated in an outpatient setting, which may reduce costs associated with admission. Owners will be advised on hygiene care, likely with guidance similar to that given to the caregivers of young patients in diapers who receive Zolgensma®, an AAV gene therapy for spinal muscular atrophy. The suggested precautions for those caregivers are to wear gloves for diaper changes, double-bag the diaper, and clean hands thoroughly afterwards with soap and water or an alcohol-based hand sanitizer^[111]. It is advised that diapers can still be disposed of in household waste.

Efficacy of liver-directed AAV gene replacement therapy in dogs

Hemophilia A

Thirty severe HA dogs without inhibitors have been reported in the literature [Table 2] with a median of 6.7 (range, 1.1-12) years of follow-up^[9,10,83,88,92,94,105]. The dogs were treated with AAV2, AAV type 6 (AAV6), AAV8, and AAV type 9 (AAV9) vectors at doses ranging from 5.4×10^{12} to 5×10^{13} vg/kg. Efficacy measurements include the endogenous factor production level achieved as well as the reduction in bleeding rate normalized over a year (annualized bleeding rate or ABR). The goal of prophylaxis is to reduce spontaneous ABR to as close to zero as possible. The median canine FVIII activity by chromogenic assay was 3.4% (range, 0.3-13.8) and the median ABR was 0.1 (range, 0-2.3) bleeds per year. The median ABR before gene therapy in the QU colony was 4 (range, 2.1-10). Although the ABR was not reported in the UNC-CH colony study, those colony dogs typically experience 4-5 bleeds per year. In the companion dog study, the median ABR was 5.2 (range, 2.6-18.9) bleeds per year. The pre-treatment ABR trends higher in the companion dog study compared to the reported ABR in the QU colony study, suggesting that real-world activity may increase the risk of bleeding in the companion animal population. As HA is an X-linked disorder, most treated HA dogs were male. Across these reports, seven homozygous females were treated with AAV2, AAV6 and AAV8 vectors at doses ranging from 6×10^{12} to 3×10^{13} vg/kg. These females had a median FVIII activity of 2.25% (range, 0.3%-12.1%) and median ABR of 0.4 (range, 0-1.4) over a median of

Table 2. Efficacy of AAV canine gene therapy in non-inhibitor hemophilia A dogs

Dog	Sex	Capsid	Transgene	Dose (vg/kg)	Follow-up (years)	FVIII activity (%)	ABR
Queens colony ^[83,94]							
ELI	F	AAV2	cFVIII-SQ	6×10^{12}	8.2	3.4	0.4
VC	M	AAV2	cFVIII-SQ	1.5×10^{13}	11	2.9	0.7
JUN*	M	AAV2	cFVIII-SQ	2.7×10^{13}	12	7.2	0.2
ALX	F	AAV6	cFVIII-SQ	1×10^{13}	10.5	8.6	0.0
MZ	M	AAV6	cFVIII-SQ	1×10^{13}	10.1	7.9	0.1
FLO	F	AAV8	cFVIII-SQ	1×10^{13}	10.5	1.8	0.1
ANG	M	AAV2	cFVIII-SQ	1.5×10^{13}	11.5	0.5	1.5
MG	F	AAV6	cFVIII-SQ	1.7×10^{13}	11.5	0.3	0.9
UNC-CH colony ^[88,92,105]							
F24	M	AAV8	Two chain	2.5×10^{13}	2.2	2.7	0.0
Woodstock	M	AAV9	Two chain	2.5×10^{13}	8.2	7.1	0.1
J60	M	AAV9	Two chain	2.5×10^{13}	9.5	4.5	0.0
Linus	M	AAV8	Two chain	1.2×10^{13}	10.1	11.3	0.3
H19	M	AAV9	Two chain	1.2×10^{13}	8.3	2.5	0.1
M06	M	AAV8	cFVIII-SQ	4×10^{13}	2.3	9.4	0.4
M50	M	AAV8	cFVIII-SQ	4×10^{13}	7.3	10.3	0.0
L51	M	AAV8	cFVIII-SQ	2×10^{13}	6.1	1.9	0.2
M66	M	AAV8	cFVIII-SQ	2×10^{13}	6	3.7	0.0
G62	F	AAV8	Two chain	1.52×10^{13}	1.10	2-2.5	1-2 events
E73	F	AAV8	Two chain	3×10^{13}	1.78	2-2.5	1-2 events
Companion dogs ^[9,10]							
PC1	M	AAV8	Two chain	5×10^{13}	8.88	2.4	1.7
PC2	M	AAV8	Two chain	5×10^{13}	8.49	1.1	0.5
PC3	M	AAV8	cFVIII-ΔF	6×10^{12}	2.56	2.1	2.3
PC5	M	AAV8	cFVIII-ΔF	6×10^{12}	5.55	12.2	0.4
PC6	M	AAV8	cFVIII-ΔF + V3	5.4×10^{12}	5.03	13.8	0.0
PC7	M	AAV8	cFVIII-ΔF + V3	6×10^{12}	4.34	5.5	0.0
PC8	M	AAV8	cFVIII-ΔF + V3	6×10^{12}	3.41	NA	0.3
PC9	M	AAV8	cFVIII-ΔF + V3	6×10^{12}	3.29	3.2	0.0
PC10	F	AAV8	cFVIII-ΔF + V3	9×10^{12}	3.86	12.1	0.0
PC11	M	AAV8	cFVIII-ΔF + V3	6×10^{12}	3.77	1.5	1.3
PC12	M	AAV8	cFVIII-ΔF + V3	6×10^{12}	3.84	2	0.0

*Transient antibody from weeks 2-9; ABR: Annualized bleed rate.

8.2 (range, 1.1-11.5) years of follow-up, which is similar to their male counterparts at 3.5% FVIII activity and ABR of 0.2.

Hemophilia B

Initial studies for *F9* gene delivery in HB dogs were conducted in skeletal muscle either via direct intramuscular injection^[61,63,80] or transvenular delivery^[85,91,112]. While these methods resulted in intramuscular expression, there was concern for immunogenicity with ectopic FIX expression. Liver-directed AAV gene

Table 3. Efficacy of AAV canine FIX gene therapy in non-inhibitor hemophilia B dogs

Dog	Sex	Age (mo)	Capsid	Vector	Dose (vg/kg)	FIX antigen (ng/mL)	FIX activity (%)	Follow-up (years)	ABR
C51 ^[86,113]	F	6.5	AAV2	LSP-cFIX	3.7×10^{11}	3.6 ± 0.5	NR	1.0	0.0
C52 ^[86,113]	M	6.5	AAV2	LSP-cFIX	2.8×10^{11}	2.9 ± 0.7	0.7 ± 0.2	3.2	0.9
		45	AAV5	LSP-cFIX-W	2.3×10^{13}	799	16.0 ± 1.7	2.5	0
C55 ^[86,113]	M	12	AAV2	LSP-cFIX-WPRE	4.6×10^{12}	218.1 ± 26.5	4	5.0	0.0
D39 ^[86,113]	F	2.8	AAV2	LSP-cFIX-WPRE	2.8×10^{12}	31.7 ± 3.2	< 0.1	2.7	2.2
		36	AAV8	LSP-cFIX-W	9.28×10^{12}	785	15.7 ± 1.2	2	0
Brad ^[82,89]	M	9	AAV2	ApoE-hAAT-cFIX	1.1×10^{12}	473	10	7.5	0.0
Semillon ^[82,89]	M	5.5	AAV2	ApoE-hAAT-cFIX	1.2×10^{12}	167	5	8.0	0.0
E34 ^[82,89]	F	5	AAV2	ApoE-hAAT-cFIX	8×10^{11}	262 ± 92	5 ± 2.5	1.0	0.0
Beech ^[82,89]	M	12	AAV2	ApoE-hAAT-cFIX	3.4×10^{12}	< 2,560	< 3	0.2	Fatal
F56 ^[84]	M	3	AAV2	LSP-cFIX-WPRE	2.2×10^{12}	< 5	NR	1.0	1.0
G10 ^[84]	M	3	AAV2	LSP-cFIX-WPRE	1.9×10^{13}	30 ± 4.84	NR	0.6	0.0
G43 ^[86]	M	7.6	AAV8	LSP-cFIX-W	5.2×10^{12}	$1,300 \pm 200$	25.9 ± 4.7	1.3	0
H12 ^[86]	F	4	AAV8	LSP-cFIX-W	5.2×10^{12}	468 ± 110	9.4 ± 2.2	1.2	0
Wick ^[72]	M		AAV8	ApoE-hAAT-cFIX-R338L (Padua)	1×10^{12}	3.33 ± 0.38	39.9 ± 5.8	1.6	0
Trex ^[72]	M		AAV8	ApoE-hAAT-cFIX-R338L (Padua)	3×10^{12}	3.81 ± 1.29	26.8 ± 5.5	3	0
PC4 ^[10]	M	56	AAV8	cFIX-WT	3×10^{12}	7,255	8.7	6.04	0.0

Age is age at gene therapy treatment. NR: Not reported; ABR: annualized bleed rate.

therapy studies in canines were consequently done first with catheterization of the portal or mesenteric veins^[82,86,89,113] before studies using peripheral intravenous infusion were conducted^[9,10,72,84]. A total of 15 severe HB dogs without pre-existing FIX inhibitors have been treated across 7 studies with AAV2, AAV type 5 (AAV5) and AAV8 vectors at doses ranging from 2.8×10^{11} to 2.3×10^{13} vg/kg [Table 3]. Over a median follow-up of 2 years (range, 0.2-13.5), these dogs express a median canine FIX activity of 9.4% (range, 0%-39.9%). Three dogs treated in early trials did not have a good response. Dog C52, treated at 2.8×10^{11} vg/kg AAV2-cFIX, expressed 0.7% FIX activity but was rescued with 2.3×10^{13} vg/kg AAV5-cFIX, resulting in 16% FIX activity activity about 3 years after the initial AAV2 vector infusion^[86,113]. Similarly, dog D39 had < 0.1% FIX activity after 2.8×10^{12} vg/kg AAV2-cFIX-WPRE vector but expressed ~16% FIX after AAV8-cFIX infused ~3 years later^[86,113]. The final dog (Beech) - whose case is complicated by pre-existing pyruvate kinase deficiency - did not respond due to development of an anti-cFIX inhibitor following gene therapy and died from a fatal abdominal hemorrhage 11 weeks after gene therapy^[82]. Of note, this is the only reported occurrence of a sustained anti-cFIX immune response with liver-directed gene therapy in HB dogs. The median ABR in HB dogs following gene therapy is 0 (range, 0-2.2), which represents a dramatic improvement in bleeding phenotype from the 6-7 bleeds per year these dogs typically experience.

Quality of life

The companion pet study did include a QOL assessment following gene therapy, albeit with a possibility of recall bias as the pre-gene therapy scoring was completed concurrently with post-gene therapy scoring^[10]. Nevertheless, there was an increase in overall QOL including an improvement in nearly all negative attributes as well as positive physical attributes such as playfulness and activities of daily living^[10]. Ongoing studies should include QOL assessments both before and at intervals after gene therapy to more formally capture improvements in companion dogs following gene therapy.

Table 4. Factor activity and inhibitor tolerance in hemophilic dogs

Dog	Inhibitor titer (BU)	Time to inhibitor eradication (weeks)	Follow-up (years)	Factor activity (%)
Hemophilia A				
Wembley	3.5	80	1.8	< 1
K01	12	5	1.9	1.5
K03	12	4	2.4	8
L44	4.5	4	1.3	1.5
PC3	21	109	2.6	2.1
Hemophilia B				
Wiley	5	4	3.5	124.2
Otis	1.2	13	2.2	47.1

Comparison between research colony and companion dogs

Gene therapy has resulted in similar increases in factor activity and decreased bleeding rates in both research colony and companion dogs with hemophilia, despite differences in their disease-causing mutations and environment. The reported UNC-CH and QU colony dogs with severe HA had the intron 22 inversion mutation, as did 7 of 11 companion dogs. Four companion dogs (PC1, PC8, PC9, PC10) had *F8* mutations that have not been previously described in dogs with hemophilia, similar to the heterogeneity of humans with severe HA. As noted above, the pre-treatment median ABR trended higher in the companion dogs (5.2) compared to the QU colony dogs (4). This is likely a reflection of their environment, with companion dogs having a more active lifestyle that increases the risk of bleeding events. The post-treatment median ABRs and FVIII activities were similar for both colony dogs (0.2% and 3.4%, respectively) and companion dogs (0.15% and 2.6%, respectively). Clinical monitoring is likely more intense for companion dogs, with owners closely observing dogs' attitude, activity, and overall demeanor for any subtle changes that could be indicative of a bleeding or other adverse event. However, colony dogs are also closely monitored by dedicated staff who care for these dogs and are unlikely to be lost to follow-up. Whether a dog is living in a research colony or the real world, it is possible for bleeding events to go unnoticed. However, it is clear that gene therapy for severe hemophilia has resulted in a marked reduction in bleeding events in both colony and companion dogs. No notable differences in the safety of AAV gene therapy have been described between companion and research dog studies.

Safety of AAV gene replacement therapy in dogs

Immune responses to transgene, vector, and capsid

Anti-transgene immune responses have largely been transient in dogs without pre-existing immunity^[83,87,92] with a notable exception in one of 4 HB dogs with a null *F9* mutation treated with a liver-directed AAV vector^[82]. For HA dogs, 1/9 in the QU colony^[83,87] and 1/8 dogs in the UNC-CH colony^[92] developed inhibitors at peak titers of 2.5-9 BU, which were eradicated by 7-9 weeks after vector injection. Notably, AAV liver-directed gene therapy has resulted in immune tolerance in dogs with pre-existing FVIII and FIX inhibitors [Table 4]^[10,72,90]. The possible mechanisms behind this tolerance have been discussed elsewhere^[114], but likely include T regulatory cells^[90,115,116]. These data largely support that liver-directed AAV gene therapy promotes tolerance to FVIII or FIX^[117,118]; indeed, a clinical trial of AAV5-FVIII is ongoing in people with HA with inhibitors^[119,120]. As discussed above, there have not been anti-capsid-directed cellular immune responses reported in hemophilic dogs treated with AAV. Of note, anti-AAV humoral immune responses against the injected capsid serotype have been robust and sustained at titers that would prohibit redosing^[94,105].

Serious adverse events

A few deaths were reported in the companion animal study, though none related to the vector itself. One dog (PC1) had neurologic deterioration at 9 years after gene therapy during an extensive trauma-induced forelimb hemorrhage at a FVIII activity of 2.4%. A second dog experienced a subdural hemorrhage 2 years after gene therapy with a FVIII activity of 2.1%. Recently, PC11 died following a presumed intracranial hemorrhage at 7 years following gene therapy; his last FVIII activity was 1.5% (Mary Beth Callan, unpublished data). These deaths emphasize the need for higher steady-state factor levels to minimize the risk of serious bleeding events as all three dogs had levels < 3%. Clinical studies in moderate HA and HB mirror the potential for increased bleeding events, and recent guidelines in human disease suggest trough levels > 3%-5% to prevent bleeding complications^[31]. This therapeutic level could be achieved using hyperactive transgenes which permit reduction of total AAV dose (thus limiting AAV-related toxicity) while enabling higher factor activity. Approaches to enhance FVIII activity are in various stages of development and evaluation^[10,121]. For FIX, the FIX-Padua variant, with ~8-fold higher activity compared to wildtype protein^[122], has demonstrated safety and efficacy in HB dogs^[72] and humans^[74,123,124], and it is the transgene of choice in most current AAV approaches.

Genotoxicity and insertional mutagenesis

Wildtype AAV can integrate into the host chromosome at a low rate into a specific site on chromosome 19 (AAVS1) *in vitro*^[125,126]. Recent studies have reported an association of AAV2 with acute hepatitis in children^[127,128], but there is no direct evidence for how AAV2 triggers hepatitis. However, recombinant AAV vectors only retain the ITRs from wildtype AAV and thus should have lower integration risk as they are largely maintained in episomal form in the host cell^[60]. Integration of recombinant AAV has been described in human liver and, to a lesser degree, cardiac tissue^[129], raising concern for tumorigenesis especially hepatocellular carcinoma. However, no causative role for wildtype^[130-132] or recombinant^[133,134] AAV integration in tumor development in humans has been clearly established.

Malignancies have been described both in human AAV trials^[135-137] and in dogs^[94,138]. One companion dog presented 3.2 years after gene therapy with fulminant multicentric lymphoma and was euthanized; integration analysis did not detect AAV in the tumor samples^[138]. Examination for vector integration and oncogenic risk has been conducted in both the QU and UNC-CH colony HA dogs^[94,105,139]. One dog in the QU colony developed a soft tissue tumor, which did not have evidence of detectable AAV copies, suggesting that it was unlikely related to vector administration^[94]. Necropsy specimens from these dogs revealed no evidence of chronic liver disease or liver tumors^[94]. Vector copies by quantitative polymerase chain reaction/reverse transcription polymerase chain reaction (qPCR/RT-PCR) were present in liver and spleen samples with canine FVIII messenger RNA only detected in the liver^[94]. More sophisticated sequencing studies for AAV integration analysis from these specimens were subsequently conducted^[139]. These specimens, obtained ~10 years from vector infusion, support that approximately 95% of the vector is retained in episomal form, with less than 5% that is integrated, with an integration frequency of 0.93 events per 1,000 cells. The majority of integration events were fragmented or rearranged and located in intergenic regions. Common integration sites were identified in proximity to 5 liver-expressed genes (*KCNIP2*, *CLIC2*, *ABCB1*, *F8* and *ALB*) but no dysregulated expression of these genes was noted. No significant clonal expansion was seen in these samples and the distribution of integration sites proximal to transcriptional start sites of cancer genes was consistent with that of a random control.

In the UNC-CH colony, there were similarly no clinical adverse events or malignancy related to AAV administration detected in any of the 9 treated dogs^[105]. Two dogs had gradual increase in FVIII expression starting at 4 years after AAV vector administration independent of transgene, vector dose, and promoter

element thus raising the question of clonal expansion and vector integration. Neither dog had any inflammatory or hepatic pathologic findings. Sequencing analyses from 20 liver specimens in 6 dogs demonstrated 1,741 putative integration sites that were largely rearranged and fragmented (as in QU colony). Integration events were favored in transcriptional units near cytosine phosphate guanine islands and modestly more frequent in cancer-associated genes. Five genes were found at integration sites that expanded in multiple dogs: *EGR2*, *EGR3*, *CCND1*, *LTO1*, and *ZNF365*; with clusters noted at *EGR2*, *EGR3*, *CCND1*, *ALB* and *DUSP1*^[105].

Notably, across both the QU and UNC-CH colonies, there were 5 overlapping integration site clusters noted (2 in chromosome 4 and 1 each in chromosomes 13, 18, and 25)^[139]. Together, these studies suggest that integration of AAV vectors is a complex phenomenon with high variability and while clonal expansion may be seen in some dogs, there is no clear association with malignancy even after 10 years of follow-up. Ongoing investigations and long-term follow-up of dogs treated with AAV vectors will further inform on the genotoxicity of recombinant AAV vectors.

Limitations of current data

While the published data to date on the efficacy and safety of gene therapy for severe hemophilia in colony and companion dogs are laudable given the inherent expense and longevity of these studies, they comprise a relatively small sample size of a total of 52 dogs: 30 HA and 15 HB with no inhibitors, and 5 HA and 2 HB with inhibitors. Furthermore, while the colonies are outbred, the research colony dogs with hemophilia have the same disease-causing mutations. Given that the severity of the *F8* mutation and the pre-AAV infusion anti-FVIII inhibitor titer may be important in predicting response to AAV gene therapy, inclusion of hemophilic dogs with different genetic backgrounds could be beneficial in future canine gene therapy studies.

FUTURE OF THE FIELD

With the commercialization of multiple AAV-based gene replacement therapies for HA and HB, preclinical research in canine models has slowed dramatically. The extremely sparse recently published research in dogs with hemophilia is focused on examining vector integration following AAV administration^[105,138,139], long-term follow-up of animals treated years ago^[10], or ways to modify gene expression after vector administration^[140]. Unfortunately, commercially available AAV gene therapy products for human patients with hemophilia cannot be used in dogs due to the development of anti-human factor inhibitors in treated canines^[52-54]. It is possible that the field will return to gene replacement studies in canines in order to solve the longevity issues seen in human patients. It is also possible that the next round of canine studies will focus on gene editing. As of yet, no studies have been published on using CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-based gene or base editing in dogs with hemophilia. Preclinical studies have been completed in mouse models of hemophilia^[141-143] and *in vitro* work has shown that human liver cell lines with a mutated canine *FIX* gene knocked in can be corrected with gene editing^[144]. However, even if AAV-based gene editing for hemophilia in research dogs and humans is developed, we expect that the ability to translate these products into widely available products for veterinary care will be similarly limited by the challenges faced by gene replacement therapies. The research-grade AAV vectors used in the canine hemophilia studies were produced by academic institutions or biotechnology companies. The vector manufacturing process is complex and time-consuming, with long turnaround times and at prohibitive costs for clinical-grade vectors (e.g., ~3 million US dollars (USD) wholesale acquisition cost for the commercial human vectors). FDA approval for gene therapy in dogs is managed by the FDA's Center for Veterinary Medicine (CVM) through the new animal drug application (NADA) process and would require veterinary studies for each vector/transgene.

CONCLUSIONS

Canine hemophilia has been an important large animal model for preclinical studies of gene therapy in hemophilia and other monogenic diseases. The major advantages over rodent models include the natural occurrence of hemophilia with genotypic heterogeneity in dogs of varying breeds, the presence of bleeding events similar to human disease, the recapitulation of inhibitor development following factor treatment, and the opportunity for long-term follow-up to inform on safety and efficacy. The studies described herein have informed the choice of vectors, transgenes, dosing, and safety in human trials, ultimately leading to the approval of AAV gene therapy vectors for HA and HB within the last 5 years. Whether and how AAV gene therapy can be brought to companion animals in clinical veterinary practice remains challenging. Overall, the cost of vector, manufacturing processes, and regulatory burden may prove too cumbersome to implement AAV gene therapy for hemophilia into clinical veterinary practice and thus limit gene therapy availability to research protocols. Nevertheless, the data shows that AAV gene therapy can be safely administered to companion animals with appropriate monitoring.

DECLARATIONS

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Authors' contributions

Conceptualized, wrote, and revised the manuscript: Doshi BS, Crudele JM, Callan MB

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