

GENE THERAPY FOR TREATING HEMOPHILIA A

INCORPORATION-BY-REFERENCE OF MATERIAL SUBMITTED IN

5 ELECTRONIC FORM

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CROSS REFERENCE TO RELATED APPLICATIONS

10 This application claims priority to US Provisional Patent Application Nos. 62/323,336, filed April 15, 2016, 62/331,807, filed May 4, 2016 and 62/428,866, filed December 1, 2016. These applications are incorporated herein by reference in their entirety.

15 1. INTRODUCTION

The application relates to embodiments useful for a gene therapy for treating hemophilia A.

2. BACKGROUND

20 Hemophilia A (HA or HemA) is the most common inherited bleeding disorder. According to the US Centers for Disease Control and Prevention, hemophilia A occurs in approximately 1 in 5,000 live births. There are about 20,000 people with hemophilia A in the US. Hemophilia A is four times as common as hemophilia B, and more than half of patients with hemophilia A have the severe form of hemophilia. HA is caused by a
25 deficiency of factor VIII (FVIII) and is well suited for a gene replacement approach, primarily because a modest increase in the level of FVIII (>1% of normal) can ameliorate the severe bleeding phenotype. Adeno-associated viral (AAV) vectors currently show the greatest promise for gene therapy applications because of their excellent safety profile and ability to direct long-term transgene expression from
30 postmitotic tissues such as the liver.

The use of AAV vectors for HA gene therapy, however, poses new challenges because of the distinct molecular and biochemical properties of human FVIII ("hFVIII"). Compared with other proteins of similar size, expression of hFVIII is highly

inefficient. Bioengineering of the FVIII molecule has resulted in improvement of the FVIII expression. For instance, the hFVIII B domain, which is not required for co-factor activity, has been deleted (BDD) and replaced by a short 14 amino acid linker (FVIII SQ) resulting in a 17-fold increase in mRNA levels over full-length wild-type FVIII and a 30% increase in secreted protein. See, Ward, Natalie J., et al. "Codon optimization of human factor VIII cDNAs leads to high-level expression." *Blood* 117.3 (2011): 798-807 and U.S. Patent No 9,393,323, also published as WO 2011/005968. Recombinant FVIII-BDD-SQ is in clinical use as a replacement recombinant FVIII product (Refacto, Wyeth Pharma; Xyntha, Pfizer).

Another obstacle to AAV-mediated gene transfer for HA gene therapy is the size of the FVIII coding sequence, which at 7.0 kb, far exceeds the normal packaging capacity of AAV vectors. Packaging of large expression cassettes into AAV vectors has been reported, but this is a highly inconsistent process resulting in low yields of vector particles with reduced infectivity and requiring a high dose that might induce liver damage. See, e.g. Sarkar, R., W. Xiao, and H. H. Kazazian. "A single adeno-associated virus (AAV)-murine factor VIII vector partially corrects the hemophilia A phenotype." *Journal of Thrombosis and Haemostasis* 1.2 (2003): 220-226; and McIntosh, Jenny, et al. "Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant." *Blood* 121.17 (2013): 3335-3344.

Thus, more efficient AAV.FVIII vectors are needed for HA treatment.

3. SUMMARY

The embodiments described herein relate to an AAV gene therapy vector for delivering normal human FVIII to a subject in need thereof, following intravenous administration of the vector resulting in long-term, perhaps 10 years or more, of clinically meaningful correction of the bleeding defect. The subject patient population is patients with moderate to severe hemophilia A. The intended vector dose is intended to deliver FVIII blood levels of approximately 3-10% or 5%. The goal for the AAV vector treatment is conversion of severe hemophilia A patients to either moderate or mild hemophilia A thus relieving such patients of the need to be on a prophylaxis regimen.

The gene therapy product described herein provides multiple important advantages to currently available prophylactic approaches to the management of severe Hemophilia A. First, preclinical results with the investigational product are consistent with its potential to achieve circulating levels of Factor VIII of 10% or more of normal, levels which would be transformative in the target patient population. Second, the product should lead to effectively constant Factor VIII blood levels, avoiding the trough levels currently seen with administration of exogenous factor. Third, by only requiring a single administration, the requirement for frequent intravenous administrations could be reduced for an extended period of time, perhaps for a decade or more.

This application provides the use of a replication deficient adeno-associated virus (AAV) to deliver a human Factor VIII (hFVIII or hF8) gene to liver cells of patients (human subjects) diagnosed with hemophilia A. The recombinant AAV vector (rAAV) used for delivering the hFVIII gene ("rAAV.hFVIII") should have a tropism for the liver (*e.g.*, a rAAV bearing an AAVhu.37 or an AAVrh.10 capsid), and the hFVIII transgene should be controlled by liver-specific expression control elements. In one embodiment, the expression control elements include one or more of the following: a transthyretin enhancer (enTTR); a transthyretin (TTR) promoter; and a polyA signal. In another embodiment, the expression control elements include one or more of the following: a shortened α 1-microglobulin/bikunin precursor (ABPS) enhancer, and enTTR; a transthyretin (TTR) promoter; and a polyA signal. In one embodiment, the expression control elements include one or more of the following: a transthyretin enhancer (enTTR); an alpha 1 anti-trypsin (A1AT) promoter; and a polyA signal. In another embodiment, the expression control elements include one or more of the following: an ABPS enhancer, and enTTR; an A1AT promoter; and a polyA signal. Such elements are further described herein.

In one embodiment, the hFVIII gene encodes a B-domain deleted (BDD) form of factor VIII, in which the B-domain is replaced by a short amino acid linker (FVIII-BDD-SQ, also referred to herein as hFVIII). In one embodiment, the FVIII-BDD-SQ protein sequence is shown in SEQ ID NO: 3. In one embodiment, the FVIII-BDD-SQ coding sequence is shown in SEQ ID NO: 1. The coding sequence for hFVIII is, in one embodiment, codon optimized for expression in humans. Such sequence may share less

than 80% identity to the native hFVIII coding sequence (SEQ ID NO: 1). In one embodiment, the hFVIII coding sequence is that shown in SEQ ID NO: 2.

In another aspect, provided herein is an aqueous suspension suitable for administration to a hemophilia A patient which includes the rAAV described herein. In some embodiments, the suspension includes an aqueous suspending liquid and about 1×10^{12} to about 1×10^{14} genome copies (GC) of the rAAV/mL. The suspension is, in one embodiment, suitable for intravenous injection. In other embodiment, the suspension further includes a surfactant, preservative, and/or buffer dissolved in the aqueous suspending liquid.

In another embodiment, provided herein is a method of treating a patient having hemophilia A with an rAAV as described herein. In one embodiment, about 1×10^{11} to about 3×10^{13} genome copies (GC) of the rAAV/kg patient body weight are delivered to the patient in an aqueous suspension.

The goal of the treatment is to functionally replace the patient's defective hFVIII via rAAV-based liver-directed gene therapy as a viable approach to treat this disease and improve response to current therapies. The embodiments described in the application are based, in part, on the development of therapeutic compositions and methods that allow for the safe delivery of efficacious doses; and improved manufacturing methods to meet the purification production requirement for efficacious dosing in human subjects.

4. BRIEF DESCRIPTION OF THE DRAWINGS

10 FIG. 1 is a schematic representation of pAAV.E03.P3.hF8co-SQ.PA75 cis plasmid.

FIG. 2 is a schematic representation of pAAV.E12.P3.hF8co-SQ.PA75 cis plasmid.

15 FIG. 3 is a schematic representation of pAAV.E03.P2.hF8co-SQ.PA75 cis plasmid.

FIG. 4 is a schematic representation of pAAV.E12.P2.hF8co-SQ.PA75 cis plasmid.

FIG. 5 shows variation in hFVIII activity prior to antibody generation in FVIII KO mice. FVIII KO mice were administered IV with 10^{10} GC of AAVrh10 vectors

expressing hFVIIIco-SQ from one of the 42 enhancer/promoter combinations. Each of the enhancer arrangements (denoted as E01-E14, Table 1) were combined with the TBG-S1 (left cohort from each grouping), A1AT (middle cohort) and TTR (right cohort) promoters. hFVIII activity (A) and anti-hFVIII IgG titers (B) were determined by

5 COATEST assay and anti-hFVIII IgG ELISA, respectively. Assays were performed on mouse plasma isolated at week 2 post-vector administration. Mice are individually plotted with mean $\pm$ SEM values for activity shown (n = 10/group).

FIG. 6 shows hFVIII activity and anti-hFVIII antibody titer at week 8 following IV vector administration in FVIII KO mice. FVIII KO mice were administered IV with

10 10^{10} GC of AAVrh10 vectors expressing hFVIIIco-SQ from one of the 42 enhancer/promoter combinations. Each of the enhancer arrangements (denoted as E01-E14, Table 1) were combined with the TBG-S1 (left cohort from each grouping), A1AT (middle cohort) and TTR (right cohort) promoters. hFVIII activity (A) and anti-hFVIII IgG titers (B) were determined by COATEST assay and anti-hFVIII IgG ELISA,

15 respectively. Assays were performed on mouse plasma isolated at week 8 post-vector administration. Mice are individually plotted with mean $\pm$ SEM values for activity shown (n = 10/group).

FIG. 7 shows hFVIII activity in FVIII KO mice following IV administration of the 42 enhancer/promoter combination vectors over time. FVIII KO mice were

20 administered IV with 10^{10} GC of AAVrh10 vectors expressing hFVIIIco-SQ from one of the 42 enhancer/promoter combinations. Each of the enhancer arrangements (denoted as E01-E14, Table 1) were combined with the TBG-S1 (left cohort from each grouping), A1AT (middle cohort) and TTR (right cohort) promoters. hFVIII activity was determined by COATEST assay on mouse plasma isolated biweekly post-vector

25 administration. Mice are individually plotted with mean $\pm$ SEM (n = 10/group).

FIG. 8 shows Anti-hFVIII antibody titers in FVIII KO mice following IV administration of the 42 enhancer/promoter combination vectors over time. FVIII KO mice were administered IV with 10^{10} GC of AAVrh10 vectors expressing hFVIIIco-SQ from one of the 42 enhancer/promoter combinations. Each of the enhancer arrangements

30 (denoted as E01-E14, Table 1) were combined with the TBG-S1 (left cohort), A1AT (middle cohort) and TTR (right cohort) promoters. Anti-hFVIII IgG titers were

determined by anti-hFVIII IgG ELISA on mouse plasma isolated biweekly. Mice are individually plotted (n = 10/group).

FIG. 9 provides comparison of hFVIII activity and anti-hFVIII antibody titer by following IV administration of the E06.TTR.hFVIIIco-SQ genome by a variety of vector capsids. FVIII KO mice were administered IV with 10^{10} GC of AAVrh10, AAV8, AAV9, AAVhu37, or AAVrh64R1 vectors expressing hFVIIIco-SQ from E06.TTR. Plasma was collected biweekly and hFVIII activity (A) and anti-hFVIII IgG titers (B) were determined by COATEST assay and anti-hFVIII IgG ELISA, respectively. Mice are individually plotted with mean $\pm$ SEM values for activity shown (n = 10/group).

FIG. 10 provides expression of hFVIII in pilot non-human primate (NHP) studies. (A) Two male rhesus macaques were administered IV with 3×10^{12} GC/kg of AAVrh10.ABP2.TBG-S1.hFVIIIco-SQ. (B) Two male cynomolgus macaques were administered IV with 3×10^{12} GC/kg of AAVhu37.ABP2.TBG-S1.hFVIIIco-SQ. Macaques were bled weekly or biweekly to evaluate hFVIII expression and the presence of antibodies against the hFVIII transgene. hFVIII expression was measured in plasma by ELISA (solid line) and values are expressed as mean $\pm$ SEM. Anti-hFVIII IgG titers were also determined in plasma by ELISA (dotted line).

FIG. 11 provides expression of hFVIII in cynomolgus macaques. Five male rhesus macaques were administered IV with 1.2×10^{13} GC/kg of one of AAVrh10.E03.TTR.hFVIIIco-SQ.PA75, AAVrh10.E12.A1AT.hFVIIIco-SQ.PA75, AAVhu37.E03.TTR.hFVIIIco-SQ.PA75, or AAVhu37.E12.A1AT.hFVIIIco-SQ.PA75. Macaques were bled biweekly to evaluate hFVIII expression in plasma by ELISA and values are expressed as mean $\pm$ SEM.

FIG. 12 shows generation of anti-hFVIII antibodies in cynomolgus macaques. Five male rhesus macaques were administered IV with 1.2×10^{13} GC/kg of one of AAVrh10.E03.TTR.hFVIIIco-SQ.PA75, AAVrh10.E12.A1AT.hFVIIIco-SQ.PA75, AAVhu37.E03.TTR.hFVIIIco-SQ.PA75, or AAVhu37.E12.A1AT.hFVIIIco-SQ.PA75. Macaques were bled biweekly to evaluate the presence of antibodies against the hFVIII transgene. Anti-hFVIII IgG titers were evaluated in plasma by ELISA. Statistical analysis is shown in FIG. 14.

FIG. 13 provides a manufacturing scheme.

FIG. 14 provides time event analysis of generation of anti-FVIII antibodies shown in FIG. 12. A statistically significant difference was seen between AAVrh.10 and AAVhu.37 using a Log-rank (Mantel-Cox) test.

FIG. 15 shows a comparison of rhCG expression levels by AAVrh10, AAV8,
5 AAV3B and AAV5 vectors (first vector injection).

FIGs. 16A-16D show rhCG vector DNA copies in liver at different time points (AAVrh10, FIG. 16A; AAV8, FIG. 16B; AAV3B, FIG. 16C; AAV5, FIG. 16D).

FIGs. 17A-17B show rhAFP levels after readministration (second vector injection) with AAV3B (FIG. 17A) or AAV5 (FIG. 17B) vectors expressing rhAFP.

10 FIGs. 18A-18B show rhAFP vector genome copies in liver (FIG. 18A, AAV3B.TBG.rhAFP; FIG. 18B, AAV5.TBG.rhAFP).

FIG. 19 shows differential AAV Nab response in macaques.

FIGs. 20A-20B provide liver vector GC (Fig. 20A) or RNA transcript levels (Fig. 20B) in liver of mice injected with the AAVrh10 enhancer/promoter vectors expressing
15 hFVIIIco IV as described in Section 6.3.8.

FIG. 21 provides hFVIII RNA transcript levels in muscle (right gastrocnemius), right testis, pancreas, right kidney, spleen, right lung, and heart of mice injected with the AAVrh10 enhancer/promoter vectors expressing hFVIIIco IV as described in Section 6.3.8.

20 FIG. 22 is a graph showing long-term stable expression of human FVIII in a cynomolgus macaque (35 months) following a single intravenous injection of AAVhu37.TBG-S1.hFVIII-SQ.PA75 at 3×10^{12} GC/kg.

FIG. 23 is a graph showing liver enzyme levels (ALT, U/mL, squares; AST, U/mL, circles) in the macaque of FIG. 22.

25 FIG. 24 is a graph showing neutralizing antibody (Nab) response to the AAVhu.37 capsid.

FIG. 25 is an alignment of the hFVIIIco sequence (SEQ ID NO: 2) vs. hFVIII native (SEQ ID NO: 1) sequence.

30 5. DETAILED DESCRIPTION

The embodiments described in the application relate to the use of a replication deficient adeno-associated virus (AAV) to deliver a human Factor VIII (hFVIII) gene to liver cells of patients (human subjects) diagnosed with hemophilia A (HA). The recombinant AAV vector (rAAV) used for delivering the hFVIII gene (“rAAV.hFVIII”) should have a tropism for the liver (*e.g.*, an rAAV bearing an AAVhu.37 or AAVrh.10 capsid), and the hFVIII transgene should be controlled by liver-specific expression control elements. In one embodiment, the expression control elements include one or more of the following: a transthyretin (TTR) enhancer; a transthyretin (TTR) promoter; and a polyA signal. Such elements are further described herein.

As used herein, “AAVhu.37 capsid” refers to the hu.37 having the amino acid sequence of GenBank, accession: AAS99285, SEQ ID NO: 17, which is incorporated by reference herein. Some variation from this encoded sequence is permitted, which may include sequences having about 99% identity to the referenced amino acid sequence in AAS99285 and US 2015/0315612 (which is incorporated herein by reference) (*i.e.*, less than about 1% variation from the referenced sequence). Methods of generating the capsid, coding sequences therefore, and methods for production of rAAV viral vectors have been described. See, *e.g.*, Gao, et al, Proc. Natl. Acad. Sci. U.S.A. 100 (10), 6081-6086 (2003) and US 2015/0315612.

As used herein, “AAVrh10 capsid” refers to the rh.10 having the amino acid sequence of GenBank, accession: AAO88201, SEQ ID NO: 18 which is incorporated by reference herein. Some variation from this encoded sequence is permitted, which may include sequences having about 99% identity to the referenced amino acid sequence in AAO88201 and US 2013/0045186A1 (*i.e.*, less than about 1% variation from the referenced sequence), provided that the integrity of the ligand-binding site for the affinity capture purification is maintained and the change in sequences does not substantially alter the pH range for the capsid for the ion exchange resin purification (as discussed further herein). Methods of generating the capsid, coding sequences therefore, and methods for production of rAAV viral vectors have been described. See, *e.g.*, Gao, et al, Proc. Natl. Acad. Sci. U.S.A. 100 (10), 6081-6086 (2003) and US 2013/0045186A1.

As used herein, the term “NAb titer” a measurement of how much neutralizing antibody (*e.g.*, anti-AAV Nab) is produced which neutralizes the physiologic effect of its

targeted epitope (e.g., an AAV). Anti-AAV NAb titers may be measured as described in, e.g., Calcedo, R., et al., Worldwide Epidemiology of Neutralizing Antibodies to Adeno-Associated Viruses. *Journal of Infectious Diseases*, 2009. 199(3): p. 381-390, which is incorporated by reference herein.

5 The terms “percent (%) identity”, “sequence identity”, “percent sequence identity”, or “percent identical” in the context of amino acid sequences refers to the residues in the two sequences which are the same when aligned for correspondence. Percent identity may be readily determined for amino acid sequences over the full-length of a protein, polypeptide, about 32 amino acids, about 330 amino acids, or a peptide
10 fragment thereof or the corresponding nucleic acid sequence coding sequencers. A suitable amino acid fragment may be at least about 8 amino acids in length, and may be up to about 700 amino acids. Generally, when referring to “identity”, “homology”, or “similarity” between two different sequences, “identity”, “homology” or “similarity” is determined in reference to “aligned” sequences. “Aligned” sequences or “alignments”
15 refer to multiple nucleic acid sequences or protein (amino acids) sequences, often containing corrections for missing or additional bases or amino acids as compared to a reference sequence. Alignments are performed using any of a variety of publicly or commercially available Multiple Sequence Alignment Programs. Sequence alignment programs are available for amino acid sequences, *e.g.*, the “Clustal Omega”, “Clustal X”,
20 “MAP”, “PIMA”, “MSA”, “BLOCKMAKER”, “MEME”, and “Match-Box” programs. Generally, any of these programs are used at default settings, although one of skill in the art can alter these settings as needed. Alternatively, one of skill in the art can utilize another algorithm or computer program which provides at least the level of identity or alignment as that provided by the referenced algorithms and programs. See, *e.g.*, J. D.
25 Thomson et al, *Nucl. Acids. Res.*, “A comprehensive comparison of multiple sequence alignments”, 27(13):2682-2690 (1999).

As used herein, the term “operably linked” refers to both expression control sequences that are contiguous with the gene of interest and expression control sequences that act in trans or at a distance to control the gene of interest.

30 A “replication-defective virus” or “viral vector” refers to a recombinant, synthetic or artificial viral particle in which an expression cassette containing a gene of interest is

packaged in a viral capsid or envelope, where any viral genomic sequences also packaged within the viral capsid or envelope are replication-deficient; *i.e.*, they cannot generate progeny virions but retain the ability to infect target cells. In one embodiment, the genome of the viral vector does not include genes encoding the enzymes required to replicate (the genome can be engineered to be "gutless" - containing only the transgene of interest flanked by the signals required for amplification and packaging of the artificial genome), but these genes may be supplied during production. Therefore, it is deemed safe for use in gene therapy since replication and infection by progeny virions cannot occur except in the presence of the viral enzyme required for replication.

10 It is to be noted that the term "a" or "an" refers to one or more. As such, the terms "a" (or "an"), "one or more," and "at least one" are used interchangeably herein.

 The words "comprise", "comprises", and "comprising" are to be interpreted inclusively rather than exclusively. The words "consist", "consisting", and its variants, are to be interpreted exclusively, rather than inclusively. While various embodiments in the specification are presented using "comprising" language, under other circumstances, a related embodiment is also intended to be interpreted and described using "consisting of" or "consisting essentially of" language.

 As used herein, the term "about" means a variability of 10% from the reference given, unless otherwise specified.

20 Unless defined otherwise in this specification, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art and by reference to published texts, which provide one skilled in the art with a general guide to many of the terms used in the present application.

5.1 Gene Therapy Vectors

25 In one aspect, a recombinant adeno-associated virus (rAAV) vector carrying the human clotting factor 8 (hF8 or hFVIII) gene is provided for use in gene therapy. The rAAV.hFVIII vector should have a tropism for the liver (*e.g.*, a rAAV bearing an AAVhu.37 or AAVrh.10 capsid) and the hFVIII transgene should be controlled by liver-specific expression control elements. The vector is formulated in a buffer/carrier suitable for infusion in human subjects. The buffer/carrier should include a component that

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prevents the rAAV from sticking to the infusion tubing but does not interfere with the rAAV binding activity *in vivo*.

5.1.1. The rAAV.hFVIII Vector

5.1.1.1. The hFVIII Sequence

5 Human coagulation factor VIII is produced as a large 330-kDa glycoprotein with the domain structure A1-A2-B-A3-C1-C2, where both the A and C domains have internal sequence homology and approximately 40% sequence identity to the A and C domains of factor V (FV), which shares the same domain structure. The B domain, which constitutes 38% of the total sequence, is dispensable for procoagulant activity. FVIII in which the B domain is deleted (BDD) and replaced by a short 14 amino acid linker (FVIII SQ) is in clinical use as a replacement recombinant FVIII product, and has been shown to result in a 17-fold increase in mRNA levels over full-length wild-type FVIII and a 30% increase in secreted protein. See, McIntosh et al, Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant, *Blood*, 121(17):3335-44 (Feb 2013) and Ward et al, Codon optimization of human factor VIII cDNAs leads to high-level expression, *Blood*, 117(3):798-807 (Jan 2011), which are incorporated herein by reference.

In one embodiment, the hFVIII gene encodes the hFVIII protein shown in SEQ ID NO: 3, which is a FVIII in which the B domain is deleted (BDD) and replaced by a short 14 amino acid linker (FVIII-BDD-SQ). Thus, in one embodiment, the hFVIII transgene can include, but is not limited to, one or more of the sequences provided by SEQ ID NO:1 or SEQ ID NO: 2 which are provided in the attached Sequence Listing, which is incorporated by reference herein. SEQ ID NO: 1 provides the cDNA for native human FVIII-BDD-SQ. SEQ ID NO: 2 provides an engineered cDNA for human FVIII-BDD-SQ, which has been codon optimized for expression in humans (sometimes referred to herein as hFVIIIco-SQ or hFVIIIco-BDD-SQ). It is to be understood that reference to hFVIII herein may, in some embodiments, refer to the hFVIII-BDD-SQ native or codon optimized sequence. Alternatively or additionally, web-based or commercially available computer programs, as well as service based companies may be used to back translate the amino acid sequences to nucleic acid coding sequences,

including both RNA and/or cDNA. See, e.g., backtranseq by EMBOSS, www.ebi.ac.uk/Tools/st/; Gene Infinity (www.geneinfinity.org/sms-/sms_backtranslation.html); ExPasy (www.expasy.org/tools/). It is intended that all nucleic acids encoding the described hFVIII polypeptide sequences are encompassed,

5 including nucleic acid sequences which have been optimized for expression in the desired target subject (e.g., by codon optimization). In one embodiment, the nucleic acid sequence encoding hFVIII shares at least 95% identity with the native hFVIII coding sequence of SEQ ID NO: 1. In another embodiment, the nucleic acid sequence encoding hFVIII shares at least 90, 85, 80, 75, 70, or 65% identity with the native hFVIII coding

10 sequence of SEQ ID NO: 1. In one embodiment, the nucleic acid sequence encoding hFVIII shares about 77% identity with the native hFVIII coding sequence of SEQ ID NO: 1. In one embodiment, the nucleic acid sequence encoding hFVIII is SEQ ID NO: 2. In another embodiment, the nucleic acid sequence encoding hFVIII shares at least 99%, 97%, 95%, 90%, 85%, 80%, 75%, 70%, or 65% identity with the hFVIII coding

15 sequence of SEQ ID NO: 1 or SEQ ID NO: 2. In another embodiment, the nucleic acid sequence encoding hFVIII is SEQ ID NO: 19. In another embodiment, the nucleic acid sequence encoding hFVIII shares at least 90, 85, 80, 75, 70, or 65% identity with the hFVIII coding sequence of SEQ ID NO: 19. In yet another embodiment, the nucleic acid sequence encoding hFVIII shares at least 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52,

20 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identity with the hFVIII coding sequence of SEQ ID NO: 1 or SEQ ID NO: 2. In yet another embodiment, the nucleic acid sequence encoding hFVIII shares at least 1, 2, 3, 4,

25 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% identity with the hFVIII coding sequence of SEQ ID NO: 19. See, Ward et al, Codon optimization of human factor VIII cDNAs leads to high-level expression, Blood, 117(3):798-807 (Jan 2011), which is incorporated herein by reference for a discussion of various variants of FVIII-SQ, including codon optimized variants.

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Codon-optimized coding regions can be designed by various different methods. This optimization may be performed using methods which are available on-line (e.g., GeneArt.), published methods, or a company which provides codon optimizing services, e.g., as DNA2.0 (Menlo Park, CA). One codon optimizing approach is described, e.g., in International Patent Publication No. WO 2015/012924, which is incorporated by reference herein. See also, e.g., US Patent Publication No. 2014/0032186 and US Patent Publication No. 2006/0136184. Suitably, the entire length of the open reading frame (ORF) for the product is modified. However, in some embodiments, only a fragment of the ORF may be altered. By using one of these methods, one can apply the frequencies to any given polypeptide sequence, and produce a nucleic acid fragment of a codon-optimized coding region which encodes the polypeptide.

A number of options are available for performing the actual changes to the codons or for synthesizing the codon-optimized coding regions designed as described herein. Such modifications or synthesis can be performed using standard and routine molecular biological manipulations well known to those of ordinary skill in the art. In one approach, a series of complementary oligonucleotide pairs of 80-90 nucleotides each in length and spanning the length of the desired sequence are synthesized by standard methods. These oligonucleotide pairs are synthesized such that upon annealing, they form double stranded fragments of 80-90 base pairs, containing cohesive ends, e.g., each oligonucleotide in the pair is synthesized to extend 3, 4, 5, 6, 7, 8, 9, 10, or more bases beyond the region that is complementary to the other oligonucleotide in the pair. The single-stranded ends of each pair of oligonucleotides are designed to anneal with the single-stranded end of another pair of oligonucleotides. The oligonucleotide pairs are allowed to anneal, and approximately five to six of these double-stranded fragments are then allowed to anneal together via the cohesive single stranded ends, and then they ligated together and cloned into a standard bacterial cloning vector, for example, a TOPO® vector available from Thermo Fisher Scientific Inc. The construct is then sequenced by standard methods. Several of these constructs consisting of 5 to 6 fragments of 80 to 90 base pair fragments ligated together, i.e., fragments of about 500 base pairs, are prepared, such that the entire desired sequence is represented in a series of

plasmid constructs. The inserts of these plasmids are then cut with appropriate restriction enzymes and ligated together to form the final construct. The final construct is then cloned into a standard bacterial cloning vector, and sequenced. Additional methods would be immediately apparent to the skilled artisan. In addition, gene synthesis is readily available commercially.

5.1.1.2. The rAAV vector

Because hFVIII is natively expressed in the liver, it is desirable to use an AAV which shows tropism for liver. In one embodiment, the AAV supplying the capsid is AAVrh.37. In another embodiment, the AAV supplying the capsid is AAVrh.10. However, any of a number of rAAV vectors with liver tropism can be used.

In a specific embodiment described in the Examples, *infra*, the gene therapy vector is an AAVhu.37 vector expressing an hFVIII transgene under control of a transthyretin promoter referred to as rAAVhu.37.TTR.hFVIII. The external AAV vector component is a serotype hu.37, $T = 1$ icosahedral capsid consisting of 60 copies of three AAV viral proteins, VP1, VP2, and VP3, at a ratio of 1:1:10. The capsid contains a single-stranded DNA rAAV vector genome.

The rAAVhu.37.TTR.hFVIII genome contains an hFVIII transgene flanked by two AAV inverted terminal repeats (ITRs). The hFVIII transgene includes an enhancer, promoter, an hFVIII coding sequence, and polyadenylation (polyA) signal. These control sequences are “operably linked” to the hFVIII gene sequences. The expression cassette may be engineered onto a plasmid which is used for production of a viral vector.

The ITRs are the genetic elements responsible for the replication and packaging of the genome during vector production and are the only viral *cis* elements required to generate rAAV. The minimal sequences required to package the expression cassette into an AAV viral particle are the AAV 5' and 3' ITRs, which may be of the same AAV origin as the capsid, or which of a different AAV origin (to produce an AAV pseudotype). In one embodiment, the ITR sequences from AAV2, or the deleted version thereof (Δ ITR), are used. However, ITRs from other AAV sources may be selected. Where the source of the ITRs is from AAV2 and the AAV capsid is from another AAV source, the resulting vector may be termed pseudotyped. Typically, an expression

cassette for an AAV vector comprises an AAV 5' ITR, the hFVIII coding sequences and any regulatory sequences, and an AAV 3' ITR. However, other configurations of these elements may be suitable. A shortened version of the 5' ITR, termed Δ ITR, has been described in which the D-sequence and terminal resolution site (trs) are deleted. In other
5 embodiments, the full-length AAV 5' and 3' ITRs are used. In one embodiment, the 5' ITR sequence is shown in SEQ ID NO: 11. In one embodiment, the 3' ITR sequence is shown in SEQ ID NO: 12.

Expression of the hFVIII coding sequence is driven from a liver-specific promoter. Because of the size of the hFVIII transgene, the use of promoter of relatively
10 small size is desirable. An illustrative plasmid and vector described herein uses the transthyretin (TTR) (also referred to herein as P3) promoter, or a modified version thereof. The TTR promoter sequence is shown in SEQ ID NO: 7. Alternatively, other liver-specific promoters may be used such as the thyroxin binding globulin (TBG) (also referred to herein as P1) promoter, or a shortened version thereof, TBG-S1, which
15 sequence is shown in SEQ ID NO: 8. Another suitable promoter is the alpha 1 anti-trypsin (A1AT), or a modified version thereon (also referred to herein as P2), shown in SEQ ID NO: 9. Other suitable promoters include human albumin (Miyatake et al., J. Virol., 71:5124-32 (1997)), humAlb; and hepatitis B virus core promoter, (Sandig et al., Gene Ther., 3:1002-9 (1996). See, e.g., The Liver Specific Gene Promoter Database,
20 Cold Spring Harbor, rulai.schl.edu/LSPD, which is incorporated by reference. Although less desired, other promoters, such as viral promoters, constitutive promoters, regulatable promoters [see, e.g., WO 2011/126808 and WO 2013/04943], or a promoter responsive to physiologic cues may be used may be utilized in the vectors described herein.

In one embodiment, the expression control sequences include one or more
25 enhancer. In one embodiment the transthyretin (enTTR) (100 bp enhancer sequence from transthyretin) is included, which sequence is shown in SEQ ID NO: 5. See, Wu et al, Molecular Therapy, 16(2):280-289, Feb. 2008, which is incorporated herein by reference. In another embodiment, the En34 enhancer is included (34 bp core enhancer from the human apolipoprotein hepatic control region), which is shown in SEQ ID NO: 4.
30 In yet another embodiment, the ABPS (shortened version of the 100 bp distal enhancer from the α 1-microglobulin/bikunin precursor [ABP] to 42 bp) enhancer is included.

Such sequence is shown in SEQ ID NO: 6. In another embodiment, more than one enhancer is present. Such combination may include more than one copy of any of the enhancers described herein, and/or more than one type of enhancer. In various embodiments, the enhancers are present in one of the following combinations:

5 Table 1: Enhancer combinations

Combination name	En34	ABPS	EnTTR	Net length (bp)
E01	1	0	0	34
E02	0	1	0	42
E03	0	0	1	100
E04	1	1	0	76
E05	0	1	1	142
E06	1	0	1	134
E07	2	0	0	68
E08	0	2	0	84
E09	0	0	2	200
E10	1	1	1	176
E11	2	0	1	168
E12	0	2	1	184
E13	1	2	0	118
E14	2	1	0	110

In one embodiment, the enhancers are combined in the following sequence: 5'-EnTTR-ABPS-En34-Promoter-3'. In another embodiment, the enhancers are combined in the following sequence: 5'-Promoter-EnTTR-ABPS-En34-3'. In one
10 embodiment, the expression control sequences include enTTR. In another embodiment, the expression control sequences include two copies of ABPS and 1 copy of enTTR.

In addition to a promoter, an expression cassette and/or a vector may contain other appropriate transcription initiation, termination, enhancer sequences, and efficient RNA processing signals. Such sequences include splicing and polyadenylation
15 (polyA) signals; sequences that stabilize cytoplasmic mRNA; sequences that enhance translation efficiency (i.e., Kozak consensus sequence); sequences that enhance protein stability; and when desired, sequences that enhance secretion of the encoded product. In one embodiment, a polyadenylation (polyA) signal is included to mediate termination of hFVIII mRNA transcripts. A polyA signal useful herein is an artificial polyA which is
20 about 75bp in size (PA75) shown in SEQ ID NO: 10. Examples of other suitable polyA

sequences include, e.g., bovine growth hormone, SV40, rabbit beta globin, and TK poly A, amongst others.

In one embodiment, the regulatory sequences are selected such that the total rAAV vector genome is about 5 to about 5.5 kilobases in size. In another
5 embodiment, the regulatory sequences are selected such that the total rAAV vector genome is about 5.1kb in size. In another embodiment, the regulatory sequences are selected such that the total rAAV vector genome is about 5.2kb in size. In another embodiment, the total rAAV vector genome is less than 5kb in size.

In one embodiment, the vector genome is nt 1 to nt 5110 of SEQ ID NO:
10 13. In one embodiment, the vector genome is nt 1 to nt 5194 of SEQ ID NO: 14. In one embodiment, the vector genome is nt 1 to nt 5138 of SEQ ID NO: 15. In another embodiment, the vector genome is nt 1 to nt 5222 of SEQ ID NO: 16.

5.1.2. rAAV.hFVIII Formulation

In one embodiment, the rAAV.hFVIII vector is provided in a pharmaceutical
15 composition which comprises an aqueous carrier, excipient, diluent or buffer. In one embodiment, the buffer is PBS. In a specific embodiment, the rAAV.hFVIII formulation is a suspension containing an effective amount of rAAV.hFVIII vector suspended in an aqueous solution containing 0.001% Pluronic F-68 in TMN200 (200 mM sodium chloride, 1 mM magnesium chloride, 20 mM Tris, pH 8.0). However,
20 various suitable solutions are known including those which include one or more of: buffering saline, a surfactant, and a physiologically compatible salt or mixture of salts adjusted to an ionic strength equivalent to about 100 mM sodium chloride (NaCl) to about 250 mM sodium chloride, or a physiologically compatible salt adjusted to an equivalent ionic concentration.

25 For example, a suspension as provided herein may contain both NaCl and KCl. The pH may be in the range of 6.5 to 8.5, or 7 to 8.5, or 7.5 to 8. A suitable surfactant, or combination of surfactants, may be selected from among Poloxamers, i.e., nonionic triblock copolymers composed of a central hydrophobic chain of polyoxypropylene (poly(propylene oxide)) flanked by two hydrophilic chains of polyoxyethylene (poly(ethylene oxide)), SOLUTOL HS 15 (Macrogol-15 Hydroxystearate), LABRASOL (Polyoxy caprylic glyceride), polyoxy 10 oleyl ether, TWEEN (polyoxyethylene
30 (Polyoxy caprylic glyceride), polyoxy 10 oleyl ether, TWEEN (polyoxyethylene

sorbitan fatty acid esters), ethanol and polyethylene glycol. In one embodiment, the formulation contains a poloxamer. These copolymers are commonly named with the letter "P" (for poloxamer) followed by three digits: the first two digits x 100 give the approximate molecular mass of the polyoxypropylene core, and the last digit x 10 gives the percentage polyoxyethylene content. In one embodiment Poloxamer 188 is selected. The surfactant may be present in an amount up to about 0.0005 % to about 0.001% of the suspension. In another embodiment, the vector is suspended in an aqueous solution containing 180 mM sodium chloride, 10 mM sodium phosphate, 0.001% Poloxamer 188, pH 7.3.

10 In one embodiment, the formulation is suitable for use in human subjects and is administered intravenously. In one embodiment, the formulation is delivered via a peripheral vein by bolus injection. In one embodiment, the formulation is delivered via a peripheral vein by infusion over about 10 minutes (± 5 minutes). In one embodiment, the formulation is delivered via a peripheral vein by infusion over about 90 minutes (± 10 minutes). In another embodiment, the formulation is delivered via a peripheral vein by infusion over about 20 minutes (± 5 minutes). In another embodiment, the formulation is delivered via a peripheral vein by infusion over about 30 minutes (± 5 minutes). In another embodiment, the formulation is delivered via a peripheral vein by infusion over about 40 minutes (± 5 minutes). In another embodiment, the formulation is delivered via a peripheral vein by infusion over about 50 minutes (± 5 minutes). In another embodiment, the formulation is delivered via a peripheral vein by infusion over about 60 minutes (± 5 minutes). In another embodiment, the formulation is delivered via a peripheral vein by infusion over about 70 minutes (± 5 minutes). In another embodiment, the formulation is delivered via a peripheral vein by infusion over about 80 minutes (± 5 minutes). However, this time may be adjusted as needed or desired. Any suitable method or route can be used to administer an AAV-containing composition as described herein, and optionally, to co-administer other active drugs or therapies in conjunction with the AAV-mediated delivery of hFVIII described herein. Routes of administration include, for example, systemic, oral, inhalation, intranasal, intratracheal, intraarterial, intraocular, intravenous, intramuscular, subcutaneous, intradermal, and other parenteral routes of administration.

In one embodiment, the formulation may contain, e.g., about 1.0×10^{11} genome copies per kilogram of patient body weight (GC/kg) to about 1×10^{14} GC/kg, about 5×10^{11} genome copies per kilogram of patient body weight (GC/kg) to about 3×10^{13} GC/kg, or about 1×10^{12} to about 1×10^{14} GC/kg, as measured by oqPCR or digital droplet PCR (ddPCR) as described in, e.g., M. Lock et al, Hum Gene Ther Methods. 2014 Apr;25(2):115-25. doi: 10.1089/hgtb.2013.131. Epub 2014 Feb 14, which is incorporated herein by reference. In one embodiment, the rAAV.hFVIII formulation is a suspension containing at least 1×10^{13} genome copies (GC)/mL, or greater, as measured by oqPCR or digital droplet PCR (ddPCR) as described in, e.g., M. Lock et al, *supra*.

In order to ensure that empty capsids are removed from the dose of AAV.hFVIII that is administered to patients, empty capsids are separated from vector particles during the vector purification process, e.g., using the method discussed herein. In one embodiment, the vector particles containing packaged genomes are purified from empty capsids using the process described in International Patent Application No. PCT/US2016/066013, filed December 9, 2016 and US Patent Appln No. 62/322,055, filed on April 13, 2016, and entitled "Scalable Purification Method for AAVrh.10", which is incorporated by reference herein. Briefly, a two-step purification scheme is described which selectively captures and isolates the genome-containing rAAV vector particles from the clarified, concentrated supernatant of a rAAV production cell culture. The process utilizes an affinity capture method performed at a high salt concentration followed by an anion exchange resin method performed at high pH to provide rAAV vector particles which are substantially free of rAAV intermediates. Similar purification methods can be used for AAVhu.37 based vectors. Other purification methods are described, e.g., in US Patent Application Nos. 62/266,347, 62/266,357, 62/322,071, 62/266,351, 62/322,083, 62/266,341, and 62/322,098, each of which is incorporated herein by reference.

While any conventional manufacturing process can be utilized, the process described herein (and in International Patent Application No. PCT/US2016/066013) yields vector preparations wherein between 50 and 70% of the particles have a vector genome, i.e., 50 to 70% full particles. Thus for an exemplary dose of 1.6×10^{12} GC/kg, and the total particle dose will be between 2.3×10^{12} and 3×10^{12} particles. In another

embodiment, the proposed dose is one half log higher, or 5×10^{12} GC/kg, and the total particle dose will be between 7.6×10^{12} and 1.1×10^{13} particles. In one embodiment, the formulation is characterized by an rAAV stock having a ratio of "empty" to "full" of 1 or less, preferably less than 0.75, more preferably, 0.5, preferably less than 0.3.

5 Briefly, in one embodiment, a method for separating AAV viral particles from AAV capsid intermediates is provided which involves: subjecting a mixture comprising recombinant AAV viral particles and AAV capsid intermediates to fast performance liquid chromatography, wherein the AAV viral particles and AAV intermediates are bound to an anion exchange resin equilibrated at a pH of about 10.0 and subjected to a
10 salt gradient while monitoring eluate for ultraviolet absorbance at about 260 and about 280, wherein the AAV full capsids are collected from a fraction which is eluted when the ratio of A260/A280 reaches an inflection point.

In one embodiment, the method further includes (a) mixing a suspension comprising recombinant AAV viral particles and AAV capsid intermediates and a Buffer
15 A comprising 20mM to 50 mM Bis-Tris propane (BTP) and a pH of about 10.0; (b) loading the suspension of (a) onto a strong anion exchange resin column; (c) washing the loaded anion exchange resin with Buffer 1% B which comprises a salt having the ionic strength of 10mM to 40 mM NaCl and BTP with a pH of about 10.0; (d) applying an increasing salt concentration gradient to the loaded and washed anion exchange resin,
20 wherein the salt gradient is the equivalent of about 10 mM to about 40 mM NaCl; and (e) collecting rAAV particles from elute obtained at a salt concentration equivalent to at least 70 mM NaCl, where the rAAV particles are at least about 90% purified from AAV intermediates. In one embodiment, this is determined by genome copies.

In one embodiment, the intermediates are eluted from the anion exchange resin
25 when the salt concentration is the equivalent of greater than about 50 mM NaCl. In still a further embodiment, Buffer A is further admixed with NaCl to a final concentration of 1M in order to form or prepare Buffer B. In yet another embodiment, the salt gradient has an ionic strength equivalent to 10 mM to about 190 mM NaCl. The elution gradient may be from 1% buffer B to about 19% Buffer B. Optionally, the vessel containing the
30 anion exchange resin is a monolith column and where Buffer A, Buffer B, and the salt gradient are in about 60 column volumes.

A stock or preparation of rAAV particles (packaged genomes) is "substantially free" of AAV empty capsids (and other intermediates) when the rAAV particles in the stock are at least about 75% to about 100%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least 99% of the rAAV in the stock and "empty capsids" are less than about 1%, less than about 5%, less than about 10%, less than about 15% of the rAAV in the stock or preparation.

In a further embodiment, the average yield of rAAV particles is at least about 70%. This may be calculated by determining titer (genome copies) in the mixture loaded onto the column and the amount presence in the final elutions. Further, these may be determined based on q-PCR analysis and/or SDS-PAGE techniques such as those described herein or those which have been described in the art.

For example, to calculate empty and full particle content, VP3 band volumes for a selected sample (*e.g.*, an iodixanol gradient-purified preparation where # of GC = # of particles) are plotted against GC particles loaded. The resulting linear equation ($y = mx + c$) is used to calculate the number of particles in the band volumes of the test article peaks. The number of particles (pt) per 20 μ L loaded is then multiplied by 50 to give particles (pt) /mL. Pt/mL divided by GC/mL gives the ratio of particles to genome copies (pt/GC). Pt/mL–GC/mL gives empty pt/mL. Empty pt/mL divided by pt/mL and x 100 gives the percentage of empty particles.

Generally, methods for assaying for empty capsids and AAV vector particles with packaged genomes have been known in the art. See, *e.g.*, Grimm et al., *Gene Therapy* (1999) 6:1322-1330; Sommer et al., *Molec. Ther.* (2003) 7:122-128. To test for denatured capsid, the methods include subjecting the treated AAV stock to SDS-polyacrylamide gel electrophoresis, consisting of any gel capable of separating the three capsid proteins, for example, a gradient gel containing 3-8% Tris-acetate in the buffer, then running the gel until sample material is separated, and blotting the gel onto nylon or nitrocellulose membranes, preferably nylon. Anti-AAV capsid antibodies are then used as the primary antibodies that bind to denatured capsid proteins, preferably an anti-AAV capsid monoclonal antibody, most preferably the B1 anti-AAV-2 monoclonal antibody (Wobus et al., *J. Virol.* (2000) 74:9281-9293). A secondary antibody is then used, one that binds to the primary antibody and contains a means for detecting binding with the

primary antibody, more preferably an anti-IgG antibody containing a detection molecule covalently bound to it, most preferably a sheep anti-mouse IgG antibody covalently linked to horseradish peroxidase. A method for detecting binding is used to semi-quantitatively determine binding between the primary and secondary antibodies,

5 preferably a detection method capable of detecting radioactive isotope emissions, electromagnetic radiation, or colorimetric changes, most preferably a chemiluminescence detection kit. For example, for SDS-PAGE, samples from column fractions can be taken and heated in SDS-PAGE loading buffer containing reducing agent (*e.g.*, DTT), and capsid proteins were resolved on pre-cast gradient polyacrylamide gels (*e.g.*, Novex).

10 Silver staining may be performed using SilverXpress (Invitrogen, CA) according to the manufacturer's instructions. In one embodiment, the concentration of AAV vector genomes (vg) in column fractions can be measured by quantitative real time PCR (Q-PCR). Samples are diluted and digested with DNase I (or another suitable nuclease) to remove exogenous DNA. After inactivation of the nuclease, the samples are further

15 diluted and amplified using primers and a TaqMan™ fluorogenic probe specific for the DNA sequence between the primers. The number of cycles required to reach a defined level of fluorescence (threshold cycle, Ct) is measured for each sample on an Applied Biosystems Prism 7700 Sequence Detection System. Plasmid DNA containing identical sequences to that contained in the AAV vector is employed to generate a standard curve

20 in the Q-PCR reaction. The cycle threshold (Ct) values obtained from the samples are used to determine vector genome titer by normalizing it to the Ct value of the plasmid standard curve. End-point assays based on the digital PCR can also be used.

In one aspect, an optimized q-PCR method is provided herein which utilizes a broad spectrum serine protease, *e.g.*, proteinase K (such as is commercially available

25 from Qiagen). More particularly, the optimized qPCR genome titer assay is similar to a standard assay, except that after the DNase I digestion, samples are diluted with proteinase K buffer and treated with proteinase K followed by heat inactivation. Suitably samples are diluted with proteinase K buffer in an amount equal to the sample size. The proteinase K buffer may be concentrated to 2 fold or higher. Typically, proteinase K

30 treatment is about 0.2 mg/mL, but may be varied from 0.1 mg/mL to about 1 mg/mL. The treatment step is generally conducted at about 55 °C for about 15 minutes, but may

be performed at a lower temperature (e.g., about 37 °C to about 50 °C) over a longer time period (e.g., about 20 minutes to about 30 minutes), or a higher temperature (e.g., up to about 60 °C) for a shorter time period (e.g., about 5 to 10 minutes). Similarly, heat inactivation is generally at about 95 °C for about 15 minutes, but the temperature may be lowered (e.g., about 70 to about 90 °C) and the time extended (e.g., about 20 minutes to about 30 minutes). Samples are then diluted (e.g., 1000 fold) and subjected to TaqMan analysis as described in the standard assay.

Additionally, or alternatively, droplet digital PCR (ddPCR) may be used. For example, methods for determining single-stranded and self-complementary AAV vector genome titers by ddPCR have been described. See, e.g., M. Lock et al, *Hum Gene Therapy Methods*, Hum Gene Ther Methods. 2014 Apr;25(2):115-25. doi: 10.1089/hgtb.2013.131. Epub 2014 Feb 14.

5.1.3 Manufacturing

The rAAV.hFVIII vector can be manufactured as shown in the flow diagram shown in FIG. 13. Briefly, cells (e.g. HEK 293 cells) are propagated in a suitable cell culture system and transfected for vector generation. The rAAV.hFVIII vector can then be harvested, concentrated and purified to prepare bulk vector which is then filled and finished in a downstream process.

Methods for manufacturing the gene therapy vectors described herein include methods well known in the art such as generation of plasmid DNA used for production of the gene therapy vectors, generation of the vectors, and purification of the vectors. In some embodiments, the gene therapy vector is an AAV vector and the plasmids generated are an AAV cis-plasmid encoding the AAV genome and the gene of interest, an AAV trans-plasmid containing AAV rep and cap genes, and an adenovirus helper plasmid. The vector generation process can include method steps such as initiation of cell culture, passage of cells, seeding of cells, transfection of cells with the plasmid DNA, post-transfection medium exchange to serum free medium, and the harvest of vector-containing cells and culture media. The harvested vector-containing cells and culture media are referred to herein as crude cell harvest.

The crude cell harvest may thereafter be subject method steps such as concentration of the vector harvest, diafiltration of the vector harvest, microfluidization

of the vector harvest, nuclease digestion of the vector harvest, filtration of microfluidized intermediate, purification by chromatography, purification by ultracentrifugation, buffer exchange by tangential flow filtration, and formulation and filtration to prepare bulk vector.

5 In one embodiment, the production plasmid is that shown in SEQ ID NO: 13. In one embodiment, the production plasmid is that shown in SEQ ID NO: 14. In one embodiment, the production plasmid is that shown in SEQ ID NO: 15. In another embodiment, the production plasmid is that shown in SEQ ID NO: 16.

10 In a specific embodiment, the methods used for manufacturing the gene therapy vectors are described in Section 8, *infra*.

5.2 Patient Population

Severe or moderate hemophilia A (HemA) patients are the chosen study population for several reasons. Severe hemophilia A patients are defined as having less than 1% of normal Factor VIII (FVIII) activity thus requiring frequent infusions of FVIII to control their bleeding diathesis. This represents a significant burden with respect to carrying on a normal life and in addition, the blood levels of FVIII go through the well-known peaks and troughs pattern, which is not optimal. The fact that FVIII blood levels in severe patients is less than 1% makes it possible to reliably measure even low to moderate increases in FVIII blood levels after rAAV.hVIII has been administered.

20 Recent clinical trials have borne out the validity of this approach. Moderate HemA patients are defined as having 1% up to 5% of FVIII levels in blood.

Patients who are candidates for treatment are preferably adult males ≥ 18 years of age, diagnosed with moderate/severe or severe hemophilia A. In one embodiment, the patient has a baseline FVIII activity $\leq 2\%$ of normal or documented history of FVIII activity $\leq 2\%$. In some embodiments, a patient < 18 years of age can be treated.

25 Candidates for treatment include subjects who have had at least 3 bleeding episodes per year that require on-demand treatment with FVIII. Other candidates for treatment include subjects who are treated with a prophylactic regimen of FVIII. Other criteria demonstrating that the subject is appropriate for treatment includes at least 100 days

30 exposure history to FVIII; no documented history of inhibitors (neutralizing antibodies)

to exogenous FVIII; no known allergic reaction to exogenous FVIII or any component of the rAAV.FVIII vector composition.

Prior to treatment, the hemophilia A patient should be assessed for NAb to the AAV serotype used to deliver the hFVIII gene (e.g, AAVhu.37 or AAVrh.10). Such NABs can interfere with transduction efficiency and reduce therapeutic efficacy. Hemophilia A patients that have a baseline serum NAb titer $\leq 1:5$ are good candidates for treatment with the rAAV.hFVIII gene therapy protocol.

Subjects may be permitted to continue their standard of care treatment(s) (e.g., recombinant FVIII therapy) prior to and concurrently with the gene therapy treatment at the discretion of their caring physician. In the alternative, the physician may prefer to stop standard of care therapies prior to administering the gene therapy treatment and, optionally, resume standard of care treatments as a co-therapy after administration of the gene therapy.

Desirable endpoints of the gene therapy regimen are an increase in FVIII activity to 3% of normal from baseline up to 52 weeks after administration of the gene therapy treatment. In one embodiment, patients achieve desired circulating FVIII levels (e.g., 5% or greater) after treatment with rAAV.hFVIII, alone and/or combined with the use of adjunctive treatments. In another embodiment, patients achieve circulating FVIII levels of 10%, 15%, 20% or greater after treatment with rAAV.hFVIII, alone and/or combined with the use of adjunctive treatments. In another embodiment, patients achieve circulating FVIII levels of 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 95%, 95% or greater after treatment with rAAV.hFVIII, alone and/or combined with the use of adjunctive treatments.

Nevertheless, patients having one or more of the following characteristics may be excluded from treatment at the discretion of their caring physician:

1. History of significant liver disease (ie, portal hypertension).
2. Significant hepatic inflammation or cirrhosis.
3. Evidence of active hepatitis B virus (HBV) or hepatitis C virus (HCV) infection.
4. History of human immunodeficiency virus (HIV) infection AND any of the following: CD4⁺ cell count <350 cells/mm³, change in antiretroviral therapy regimen

within 6 months prior to Day 0, or plasma viral load >200 copies/ml, on 2 separate occasions, as measured by PCR.

5. Anti-AAVhu.37 (or anti-AAVrh10, as appropriate) neutralizing antibody titer >1:5 or $\geq 1:10$.

5 6. Participation (current or previous) in another gene therapy study.

7. Participation in another investigational medicine study within 3 months before screening.

In other embodiments, a caring physician may determine that the presence of one or more of these physical characteristics (medical history) should not preclude treatment as provided herein.

10

5.3. Dosing & Route of Administration

In one embodiment, the rAAV.hFVIII vector is delivered as a single dose per patient. In another embodiment, the rAAV.hFVIII vector is delivered as multiple doses per patient. In a further embodiment, the rAAV.hFVIII vector is delivered as two doses per patient. In one embodiment, the subject is delivered the minimal effective dose (MED) (as determined by preclinical study described in the Examples herein). As used herein, MED refers to the rAAV.hFVIII dose required to achieve 5% of normal Factor VIII activity.

15

20 As is conventional, the vector titer is determined on the basis of the DNA content of the vector preparation. In one embodiment, quantitative PCR or optimized quantitative PCR as described in the Examples is used to determine the DNA content of the rAAV.hFVIII vector preparations. In one embodiment, digital droplet PCR as described in the Examples is used to determine the DNA content of the rAAV.hFVIII vector preparations. In one embodiment, the dosage is about 1×10^{11} genome copies (GC)/kg body weight to about 1×10^{13} GC/kg, inclusive of endpoints. In one embodiment, the dosage is 5×10^{11} GC/kg. In another embodiment, the dosage is 5×10^{12} GC/kg. In specific embodiments, the dose of rAAV.hFVIII administered to a patient is at least 5×10^{11} GC/kg, 1×10^{12} GC/kg, 1.5×10^{12} GC/kg, 2.0×10^{12} GC/kg, 2.5×10^{12} GC/kg, 3.0×10^{12} GC/kg, 3.5×10^{12} GC/kg, 4.0×10^{12} GC/kg, 4.5×10^{12} GC/kg, 5.0×10^{12} GC/kg, 5.5×10^{12} GC/kg, 6.0×10^{12} GC/kg, 6.5×10^{12} GC/kg, 7.0×10^{12} GC/kg, or 7.5×10^{12} GC/kg.

25

30

Also, the replication-defective virus compositions can be formulated in dosage units to contain an amount of replication-defective virus that is in the range of about 1.0×10^9 GC to about 1.0×10^{15} GC. As used herein, the term "dosage" can refer to the total dosage delivered to the subject in the course of treatment, or the amount delivered in a single (of multiple) administration.

In another embodiment, the composition is readministered at a later date. Optionally, more than one readministration is permitted. Such readministration may be with the same type of vector or a different viral vector as described herein. In one embodiment, the vector is readministered about 6 months after the first administration.

10 In another embodiment, the vector is readministered about 1 year after the first administration. In another embodiment, the vector is readministered about 2 years after the first administration. In another embodiment, the vector is readministered about 3 years after the first administration. In another embodiment, the vector is readministered about 4 years after the first administration. In another embodiment, the vector is

15 readministered about 5 years after the first administration. In another embodiment, the vector is readministered about 6 years after the first administration. In another embodiment, the vector is readministered about 7 years after the first administration. In another embodiment, the vector is readministered about 8 years after the first administration. In another embodiment, the vector is readministered about 9 years after

20 the first administration. In another embodiment, the vector is readministered about 10 years or more after the first administration.

In one embodiment, the dosage is sufficient to increase the Factor VIII levels in the patient to 1% of normal. In one embodiment, the dosage is sufficient to increase the Factor VIII levels in the patient to 2% of normal. In one embodiment, the dosage is

25 sufficient to increase the Factor VIII levels in the patient to 3% of normal. In another embodiment, the dosage is sufficient to increase the Factor VIII levels in the patient to 4% of normal. In another embodiment, the dosage is sufficient to increase the Factor VIII levels in the patient to 5% of normal. In another embodiment, the dosage is sufficient to increase the Factor VIII levels in the patient to 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17,

30 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95% or greater of normal.

In some embodiments, rAAV.hFVIII is administered in combination with one or more therapies for the treatment of hemophilia A, such as administration of recombinant FVIII.

5.4. Measuring Clinical Objectives

5 Measurements of efficacy of treatment can be measured by transgene expression and activity as determined by plasma Factor VIII levels and Factor VIII activity. Further assessment of efficacy can be determined by clinical assessment of replacement Factor VIII requirements and frequency of spontaneous bleeding episodes. Such assessments may be conducted twice a week for 4 weeks after the administration of the product,
10 weekly from week 6 to week 12, monthly throughout the remainder of the first year and at 6 month intervals for a total period of 5 years.

 Safety of the gene therapy vector after administration can be assessed by the number of adverse events, changes noted on physical examination, and/or clinical laboratory parameters assessed at multiple time points up to about 52 weeks post vector
15 administration. Although physiological effect may be observed earlier, e.g., in about one week, in one embodiment, steady state levels expression levels are reached by about 12 weeks. The following assessments may be conducted twice a week for 4 weeks after the administration of the product, weekly from week 6 to week 12, monthly throughout the remainder of the first year and at 6 month intervals for a total period of 5 years. Such
20 assessments include:

- a. Physical examination
- b. ECG
- c. Biochemical assessment: Serum electrolytes, BUN, creatinine, calcium, phosphate, total protein, albumin, LDH, CPK, AST, ALT, alkaline phosphatase,
25 bilirubin
- d. Hematological assessment: CBC and differential, coagulation profile
- e. Urinalysis
- f. Immunological assessment:
- g. Serological response to hu.37 capsid (or rh.10 capsid, as appropriate) and to
30 Factor VIII

h. T cell response to hu.37 capsid (or rh.10 capsid, as appropriate) and Factor VIII antigens

i. Assessment of vector DNA; qPCR measurements in plasma, urine and saliva.

hFVIII increase achieved with rAAV.hFVIII administration can be assessed as a
5 defined percent change in hFVIII at about 12 weeks, or at other desired timepoints,
compared to hFVIII levels of a patient not having hemophilia A, i.e., so-called normal
hFVIII levels of about 100%. In another embodiment, the change is compared to the
patient's baseline hFVIII levels. In one embodiment, the desired efficacy is an increase
in the Factor VIII levels in the patient to 3% of normal. In one embodiment, the desired
10 efficacy is an increase in the Factor VIII levels in the patient to 4% of normal. In one
embodiment, the desired efficacy is an increase in the Factor VIII levels in the patient to
5% of normal. In one embodiment, the desired efficacy is an increase in the Factor VIII
levels in the patient to 6% of normal. In one embodiment, the desired efficacy is an
increase in the Factor VIII levels in the patient to 7% of normal. In one embodiment, the
15 desired efficacy is an increase in the Factor VIII levels in the patient to 8% of normal. In
one embodiment, the desired efficacy is an increase in the Factor VIII levels in the
patient to 9% of normal. In another embodiment, the dosage is sufficient to increase the
Factor VIII levels in the patient to 10% of normal. In another embodiment, the dosage is
sufficient to increase the Factor VIII levels in the patient to 15% of normal. In another
20 embodiment, the dosage is sufficient to increase the Factor VIII levels in the patient to
20% or greater of normal. In one embodiment, coagulation panels are performed as a
part of standard testing to infer FVIII activity.

As used herein, the rAAV.hFVIII vector herein "functionally replaces" or
"functionally supplements" the patients defective FVIII with active FVIII when the
25 patient expresses a sufficient level of FVIII to achieve at least one of these clinical
endpoints. Expression levels of hFVIII which achieve as low as about 1% to less than
100% of normal wild-type clinical endpoint levels in a non-hemophilia patient may
provide functional replacement.

In one embodiment, expression may be observed as early as about 8 hours to
30 about 24 hours post-dosing. One or more of the desired clinical effects described above
may be observed within several days to several weeks post-dosing.

Long term (up to 260 weeks) safety and efficacy can be assessed after rAAV.hFVIII administration.

In one aspect, a regimen for delivery of a hFVIII gene product to a human patient is provided. The regimen comprises (a) delivery of a first rAAV.hFVIII vector
5 comprising an expression cassette as described herein; and (b) delivery of a second rAAV.hFVIII vector comprising an expression cassette as described herein, wherein the first recombinant AAV vector or the second AAV vector has an AAV3B capsid. The sequence of AAV3B is shown in SEQ ID NO: 20 and Accession No. AAB95452.1. In one embodiment, the other of the first or the second AAV vector has an rh.10 capsid. In
10 another embodiment, the other of the first or the second AAV vector has an AAVhu.37 capsid. Such regimens are described in International Patent Application No. PCT/US16/42472, which is incorporated herein by reference.

The viral vectors described herein may be used in preparing a medicament for delivering hFVIII to a subject (e.g., a human patient) in need thereof, supplying
15 functional hFVIII to a subject, and/or for treating hemophilia A disease.

In another aspect, an rAAV.hFIII vector as described herein is provided for use in treating hemophilia A. In one embodiment, multiple doses are provided for use in treating hemophilia A. In another aspect, an rAAV.hFIII vector as described herein is provided for the manufacture of a medicament for treating hemophilia A.

In one embodiment, a second administration of a rAAV.hFVIII vector is given.
20 In one embodiment, the rAAV.hFVIII vector of the second administration has the same AAV capsid as provided with the first dosage. In one embodiment, the rAAV.hFVIII vector of the second administration has an AAVrh.10 capsid. In another embodiment, the rAAV.hFVIII vector of the second administration has a different AAV capsid as the
25 vector of the first dose. In one embodiment, the rAAV.hFVIII vector of the second administration has a tropism for liver. In one embodiment, the rAAV.hFVIII vector of the second administration has an AAV3B capsid.

In a further aspect, the invention involves targeting hepatocytes of the patient.

In one aspect, the delivery of the first rAAV and the second rAAV are temporally
30 separated by at least about one month, at least about three months, or about 1 year to about 10 years.

The following examples are illustrative only and are not intended to limit the present invention.

EXAMPLES

5 **6. Example 1: Preclinical testing**

6.1 hFVIII vector

Unlike human factor FIX (hFIX), the cDNA for hFVIII is much larger and adjustments need to be made to fit this transgene into the standard AAV genome. As the B Domain Deleted (BDD) hFVIII transgene is 1457 amino acids and with the inclusion
10 of other necessary elements for transcription, an AAV vector is still at the limit of its packing capacity. Therefore, steps have been taken to reduce the size of the other elements, including the transgene expression control elements.

In order to restrict expression of hFVIII to the liver while keeping the size of the elements as small as possible several strong liver-specific promoters were shortened and
15 combined, with combinations of up to three liver-specific enhancer sequences, to generate 42 enhancer/promoter combinations. hFVIII activity and immunogenicity of the transgene were evaluated in FVIII KO mice following administration of AAV vectors.

6.1.1 AAV vector production for pre-clinical testing

20 42 plasmids were generated containing one of the enhancer/promoter combinations. 14 enhancer combinations were generated using three enhancer sequences; En34 (34 bp core enhancer from the human apolipoprotein hepatic control region), ABPS (shortened version of the 100 bp distal enhancer from the α 1-microglobulin/bikunin precursor [ABP] to 42 bp), and EnTTR (100 bp enhancer
25 sequence from transthyretin). The number of enhancer combinations was restricted due to the total ITR-ITR size and by combination of the enhancers in the following sequence: 5'-EnTTR-ABPS-En34-Promoter-3'. Table 1. Each of the 14 enhancer combinations were inserted upstream of one of three promoters; TBG-S1 (P1, shortened version of the liver-specific thyroxine binding globulin or TBG promoter), A1AT (P2, modified
30 SERINA1 [α 1-antitrypsin] promoter), and TTR (P3, transthyretin promoter). The resulting constructs were designed to express a codon-optimized version of the human

factor VIII protein where the B domain is deleted and replaced by a short 14 amino acid linker, hFVIIIco-SQ (SEQ ID NO: 2).

All AAV vectors were produced as described in Gao G, Lu Y, Calcedo R, et al. Biology of AAV serotype vectors in liver-directed gene transfer to nonhuman primates. *Mol Ther.* 2006;13(1):77-87, which is incorporated herein by reference. Briefly, plasmids expressing hFVIII from one of the 42 enhancer/promoter combinations were packaged with the AAVrh10 viral capsid. Plasmids expressing hFVIII from E06.TTR were also packaged in AAV8, AAV9, AAVhu37, and AAVrh64R1 viral capsids.

6.1.2 SDS-PAGE analysis of vectors

The AAVrh10 enhancer/promoter combination vector lots used in the study were subjected to purity assessment by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described in Lock M, Alvira M, Vandenberghe LH, et al. Rapid, simple, and versatile manufacturing of recombinant adeno-associated viral vectors at scale. *Hum Gene Ther.* 2010;21(10):1259-1271, which is incorporated herein by reference. Briefly, denatured and reduced vector samples containing 5×10^9 GC were loaded for SDS-PAGE. Proteins were stained by SYPRO ruby stain (Invitrogen, Carlsbad, CA, USA) following fixation, visualized, and then quantified using Syngene imaging analysis system and GeneTool software (Syngene, Frederick, MD). The percent purity of the capsid (VP1, VP2, and VP3 proteins indicated over total protein) was calculated. The percentage purity of the 42 vectors ranged from 29% (AAVrh10.E12.P3) to 100%, with a mean purity of 90% (data not shown).

6.1.3 Mice

Breeding pairs of FVIII KO mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and a colony was maintained at the University of Pennsylvania's Translational Research Laboratories housed under specific pathogen-free conditions. All animal procedures and protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania. Male FVIII KO at 6-12 weeks of age were injected IV with 10^{10} GC of vector per mouse into the tail vein. Vector was diluted in phosphate buffered saline (PBS) and 100 μ l of the vector dilution was injected. Plasma was collected biweekly by retro-orbital bleeds into sodium citrate collection tubes.

6.1.4 Determination of hFVIII activity

hFVIII activity was measured in plasma by COATEST SP4 kit according to the manufacturer's protocol (DiaPharma, OH, USA). At week 2 post-injection, mice showed a vast range in hFVIII activity from 0.12 - 2.12 IU/ml (FIG. 5).

5 Five constructs demonstrated significantly increased activity levels over the others; E03.TTR, E05.A1AT, E05.TTR, E06.TTR, and E12.A1AT (FIG. 5A). The variation in hFVIII activity levels seen at week 2 was prior to the generation of antibodies to the transgene (FIG. 5B). Therefore, there were construct-dependent significant differences in activity levels.

10 6.1.5 Detection of anti-hFVIII IgG in mouse plasma

IgG antibodies against hFVIII in mouse plasma were measured by ELISA, where all reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. ELISA plates were coated with 1 µg/ml BDD-hFVIII-SQ (Xyntha, Wyeth Pharmaceuticals Inc., Dallas, TX, USA) in 0.1 M carbonate buffer (pH 9.6) and
15 incubated overnight at 4°C. Wells were washed five times with 0.05% Tween 20 in PBS and blocked with 5% non-fat milk (Bio Rad, Hercules, CA, USA) in PBS for one hour at room temperature. Following removal of the blocking buffer, plasma samples diluted in 5% non-fat milk were added to the plates and incubated for one hour at room temperature. Plasma samples from naïve mice were used as the control. Plates were
20 then washed five times and HRP-conjugated anti-mouse IgG was added at a 1:1000 dilution in non-fat milk. Following incubation at room temperature for 90 minutes, plates were washed eight times and 3,3',5,5'-tetramethylbenzidine (TMB) was added for detection. The reaction was stopped after 5 minutes at room temperature using 2N sulfuric acid and plates were read at 450 nm using a BioTek µQuant plate reader
25 (Winooski, VT, USA).

FVIII KO mice showed antibody generation to hFVIII at week 4 post-vector administration and by week 8 mice in most of the 42 vector groups had detectable anti-hFVIII IgG levels with a corresponding decrease in hFVIII activity levels (FIG. 6). Time courses of hFVIII activity and antibody generation quantified by titer are presented
30 in FIG.s 7 and 8, respectively. More than 50% of mice injected with constructs E05.A1AT, E10.A1AT, and all promoters with the E06 enhancer combination had

antibodies to the transgene by week 8. However, antibodies to the transgene were not seen in all groups. For 6 out of the 42 vector groups (E11.TTR, E13.TBG-S1, E13.A1AT, and all constructs using E01), no hFVIII antibodies were detected at week 8 (FIG. 6) and two groups had no detectable antibodies throughout the duration of the 12 week study (E01.A1AT and E11.TTR). Time to event analysis for the generation of antibodies to hFVIII was also performed. Mice injected with vectors using E01.A1AT, E11.TTR, E01.TBG-S1, E11.A1AT, and E13.TBG-S1 for expression had the longest time to antibody expression, whereas constructs containing E06.TTR, E06.A1AT, E05.A1AT, E09.TBG-S1, and E14.TTR had the shortest time to antibody expression.

6.1.6 Statistical analysis

To identify similar treatment groups according to hFVIII activity in plasma at week 2, the data were analyzed using a single fixed factor ANOVA model with Tukey post hoc tests to identify group mean activity levels that differ from one another. Time to event analysis was performed for the generation of antibodies against hFVIII.

6.1.9 Comparison of activity and immunogenicity by a variety of AAV capsids

Next the differences in activity levels were determined and potential contribution to immunogenicity by the AAV capsid used was ascertained. For this study the most immunogenic genome from the previous studies was selected – E06.TTR. Interestingly, this construct produced significantly higher expression than the majority of the other constructs at week 2 but in the following weeks antibodies were generated against the hFVIII transgene in 80% of injected mice.

The same vector genome using the E06 enhancer combination with the liver-specific TTR promoter was packaged with one of five AAV capsids; AAVrh10, AAV8, AAV9, AAVhu37 and AAVrh64R1. Again, FVIII KO mice were injected IV at a dose of 10^{10} GC per mouse and plasma hFVIII activity levels and anti-hFVIII IgG titers were followed throughout a 12 week study. Stark differences in expression and immunogenicity of the transgene were seen based on the AAV vector used for gene transfer (FIG. 9). At week 2 post-vector administration, hFVIII activity in plasma varied from 0.51 IU/ml following AAVrh64R1 administration to 1.26 IU/ml with AAVrh10 (FIG. 9A). During the course of the study, several of the mice generated anti-hFVIII

antibodies ranging from 20% of mice administered with AAV8 or AAV9 vectors to 63% of mice that received AAVrh10 (FIG. 9B). Therefore, even with a highly immunogenic enhancer and promoter combination, the immune response to the transgene can vary based on the AAV capsid used for gene transfer.

5 **6.1.10 Discussion**

Previous studies in FVIII KO mice where the HLP promoter was used for expression did not detect antibodies to the transgene throughout the duration of the study. The HLP promoter sequence is similar to that of the E01.A1AT enhancer/promoter combination, where mice administered with this AAVrh10 vector had no detectable
10 antibodies throughout the duration of the 12 week study. Unfortunately, activity from this vector in FVIII KO mice was relatively low with only 0.189 IU/ml detectable in plasma at week 2 post-vector administration and a peak level of 0.303 IU/ml at week 6.

Interestingly, the AAV capsid used for gene transfer of the same transgene cassette significantly influenced both immunogenicity to hFVIII and peak hFVIII
15 activity. In order to study the effect of capsid on generation of anti-hFVIII antibodies, the most immunogenic transgene cassette was used (E06.TTR). The five vector capsids studied could be divided into two groups; those were $\leq 20\%$ of mice generated anti-hFVIII antibodies (AAV8 and AAV9) and those were $> 20\%$ of mice developed a humoral immune response (AAVrh10, AAVhu37, and AAVrh64R1). The tolerance
20 towards the hFVIII transgene associated with the AAV8 capsid is perhaps unsurprising due to previous studies that demonstrate that IV delivery of this capsid may activate transgene-specific regulatory T cells in the context of the tolerogenic nature of the liver. In addition, we have previously shown that there were no detectable FIX inhibitors following IM injection of AAV8 in hemophilia B mice. Therefore, even with a highly
25 immunogenic enhancer and promoter combination, the immune response to the transgene can vary based on the AAV capsid used for gene transfer.

hFVIII activity levels in plasma following administration of the five different AAV capsids also varied significantly from 0.51 IU/ml with AAVrh64R1 to 1.26 IU/ml with AAVrh10 at week 2 post-vector administration. Expression from the AAVrh10
30 vector was significantly elevated compared to AAV8 and coincided with the generation of anti-hFVIII antibodies in 63% of mice. This capsid comparison study was performed

using a highly immunogenic transgene cassette and likely does not model that seen in humans where ~30% of hemophilia A patients generate antibodies to the recombinant protein. Therefore, the higher expression levels produced following administration of the AAVrh10 vector may be more beneficial to the clinical situation where a lower dose of vector would be required for significant improvements in the incidence of bleeding events and supplementation with the recombinant hFVIII protein.

From the results of this study, the E03.TTR (FIG. 1; SEQ ID NO: 13), E03.A1AT (FIG. 3; SEQ ID NO: 15), E12.TTR (FIG. 2; SEQ ID NO: 14) and E12.A1AT (FIG. 4; SEQ ID NO: 16) constructs were selected for further testing.

6.2 Dosage Studies

6.2.1 Studies in FVIII KO mice to inform on the approximate MED

FVIII KO mice in C57BL/6 and 129 background receive a tail vein injection of one of four vector doses of AAVhu37.E03.TTR.hFVIIIco-SQ.PA75. Such vector doses are 5×10^{10} GC/kg, 5×10^{11} GC/kg, 5×10^{12} GC/kg, and 5×10^{13} GC/kg. A cohort of animals receiving control article only (vehicle buffer) is included as a vehicle control. After vector administration, the animals are monitored daily for general observations. Blood is collected from the animals at the appropriate time points to capture the hFVIII activity levels. Animals in subset A are sacrificed on day 60 after dosing, animals in subset B are sacrificed on day 28 after dosing, and animals in subset C are sacrificed on day 3 after dosing. Blood is also collected at necropsy for a serum chemistry panel and hematology. Sacrificed animals will be necropsied; the organs, such as right Inguinal lymph node, right testis, pancreas, duodenum, colon, brain, right gastrocnemius muscle, stomach, right kidney, right lung, spleen, heart, liver and gross lesions if any, are harvested for biodistribution and histopathology examinations. Total cellular DNA and RNA are extracted for mice that received the highest dose of vector and those that received the control article. qPCR and RT-qPCR assays are performed on the extracted DNA/RNA to measure vector genome copies and transcript levels in the organs, respectively. The efficacy of the test article is determined by the hFVIII protein activity levels in plasma by COATEST assay. Also, anti-hFVIII antibodies are monitored by anti-hFVIII IgG ELISA assay and the extrinsic pathway of coagulation is evaluated by prothrombin time (PT) assay.

6.3 Studies in non-human primates

6.3.1 Expression studies in NHP

The primary objective of this non-GLP study is to evaluate the potential vector related toxicity and biodistribution in NHP.

5 Male rhesus and cynomolgus macaques were used for this study. Only male animals were used in the study since hemophilia A is an X-linked genetic disorder. All macaques had NAb titers of <1:5 at the start of the studies determined as described previously (CALCEDO et al. (2009) Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. J Infect Dis, 199, 381-90). Prior to vector administration, 10 macaques were anesthetized with a mixture of ketamine (10–15 mg/kg) and dexmedetomidine (0.05–0.10 mg/kg) injected IM. Macaques were administered with vectors IV via the saphenous vein. Blood samples were taken prior to the initiation of the study and biweekly during the study via venipuncture of the femoral vein. All clinical pathology tests on blood samples were conducted by Antech Diagnostics (Irvine, 15 CA), including complete blood counts and differentials, complete clinical chemistries, and coagulation panels.

Pilot studies for expression of hFVIII in NHPs were performed. Two rhesus macaques and two cynomolgus macaques were administered IV with 3×10^{12} GC/kg of AAVrh10 (FIG. 10A) and AAVhu37 (FIG. 10B) vectors expressing hFVIII from the 20 ABP2.TGB-S1 enhancer/promoter combination, respectively. High peak expression was seen in all animals but by weeks 6-8. A humoral immune response to hFVIII in macaques injected with AAVrh10 was seen. The development of anti-hFVIII antibodies was delayed in one animal that received AAVhu37, occurring at 12 week post-vector administration, and did not develop throughout the course of the study for the other 25 animal. The macaque which received AAVhu37.TBG-S1.hFVIII-SQ.PA7 was followed for 35 months post-injection (FIGs 22 to 24).

Based on the FVIII KO mouse studies and this small pilot rhesus macaque study, two of the original 42 enhancer/promoter combinations were selected for further evaluation in cynomolgus macaques, using two different Clade E capsids for expression.

6.3.2 Further studies in NHP

Subsequently 20 male cynomolgus macaques were dosed with one of four vectors; AAVrh10.E03.TTR.hFVIIIco-SQ.PA75, AAVrh10.E12.A1AT.hFVIIIco-SQ.PA75, AAVhu37.E03.TTR.hFVIIIco-SQ.PA75, and AAVhu37.E12.A1AT.hFVIIIco-SQ.PA75 (n = 5 macaques per vector). Vector was IV administered at a dose of 1.2×10^{13} GC/kg (based on middle oqPCR titer). With one capsid plus enhancer/promoter combination, peak expression of 37% of normal FVIII levels was seen at week 2 post-vector administration, which then plateaued at 20% of normal (FIG. 11). While antibodies to the hFVIII were detected in the majority of macaques by week 8, antibodies remained undetectable in two animals at week 30 post-vector administration (FIG. 12). Methods discussed below. By using time to event analysis for the generation of antibodies, it was determined that there was a significant difference between AAVrh10 and AAVhu37 (FIG. 14).

6.3.3 Determination of hFVIII expression in NHP plasma

hFVIII expression was measured by ELISA, where all reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. ELISA plates were coated with anti-hFVIII IgG (Green Mountain Antibodies, VT, USA) at a 1:500 dilution in 0.1 M carbonate buffer (pH 9.6) and incubated overnight at 4°C. Wells were washed four times with 0.1% Tween 20 in PBS and blocked with 5% non-fat milk (Bio Rad, Hercules, CA, USA) in PBS for one hour at room temperature. Following removal of the blocking buffer, plasma samples diluted in 5% non-fat milk were added to the plates and incubated for one hour at room temperature. Plates were then washed four times and anti-hFVIII IgG (ThermoFisher Scientific, MA, USA) was added at a 1:1000 dilution in non-fat milk. Following incubation for one hour at room temperature plates were washed four times and HRP-conjugated anti-sheep IgG was added at a 1:1000 dilution in non-fat milk. Following incubation at room temperature for 90 minutes, plates were washed five times and 3,3',5,5'-tetramethylbenzidine (TMB) was added for detection. The reaction was stopped after 5 minutes at room temperature using 2N sulfuric acid and plates were read at 450 nm using a BioTek μ Quant plate reader (Winooski, VT, USA). FIG. 11.

Results of long-term stable expression of human FVIII in a cynomolgus macaque (35 months) following a single intravenous injection of AAVhu37.TBG-S1.hFVIII-

SQ.PA75 are shown in FIG 22. Results show stable expression until necropsy at 35 months. Results of liver enzyme testing are shown in FIG. 23 Results show that liver enzyme levels were within the normal range, except for transient elevation after liver biopsy. Neutralizing antibody (Nab) response to the AAVhu.37 capsid is shown in FIG. 24.

Single cell technology was used to detect AAV vector DNA and RNA in the hepatocytes of NHP M11269, discussed above, who received intravenous administration of AAVhu37.TBG-S1.hFVIII-SQ.PA75 at 3E12 GC/kg. Single cell hepatocytes were evaluated for presence of AAV genomes and expression. 150 weeks post vector, hepatocytes were isolated from a liver wedge by perfusing with a mixture of collagenase/protease. Single cells were sorted into individual wells of a 96 well plate. Cells from one 96 well plate were whole genome amplified (WGA) and AAV genomes in cells quantified by digital PCR using a probe against human FVIII. A second 96 well plate was whole-transcriptome amplified (WTA) for evaluating FVIII expression.

Results shown below in Table 3. AAV genomes could be detected in ~25% of the single cells evaluated. RNA expression was only detected in ~4% or 20% of the cells that take up DNA. RNA expressing cells could be stratified into two types as low and high. It is unclear why a substantial number of cells take up vector but fail to express the transgene. These results were corroborated by in situ hybridization studies performed by CMC core (data not shown).

Table 3: Percent DNA and RNA Positive Single Cells

Single cell (w152)	Sample	Positives/total	Percent positive
	DNA	23/96	24%
	RNA	4/96	4%
	RNA/DNA positive cells	4/23	17%

liver homogenate	Sample	w42 biopsy	w152 necropsy
	DNA (GC/cell)	10	7.6 +/-1.1
	hFVIII RNA (copies ug RNA)	4.4 +/- 1.1 E4	6.4 +/- 0.7E4

6.3.4 Detection of anti-hFVIII IgG in NHP plasma

IgG antibodies against hFVIII in NHP plasma were measured by ELISA as described previously, with the exception that HRP-conjugated anti-NHP IgG was added at a 1:2000 dilution in non-fat milk for detection (FIG. 12).

5 6.3.5 Detection of Bethesda titer in NHP plasma

Inhibitory antibody against human FVIII was measured by Nijmegen modified Bethesda assay (Giles *et al.*, 1998). One Bethesda unit represents inhibition of coagulation activity of normal human plasma by 50%.

6.3.6 Liver biopsies

10 Two NHPs from each group received a liver biopsy at week 5 post-vector administration performed by mini-laparotomy. Selection of animals was based on hFVIII expression in plasma at week 4. The first animal selected was the animal with the median hFVIII level, the second animal selected was the animal with hFVIII level closest or second closest (if the closest one is the median one) to the mean level.
15 Samples of liver tissue were taken for histopathology, vector biodistribution, and transgene mRNA analysis. On day 3 post-liver biopsy, blood was taken for complete blood counts and differentials, complete clinical chemistries, and coagulation panels.

6.3.7 Immunosuppression protocol

An immunosuppression regimen was initiated as required in animals where the
20 ability to detect hFVIII expression was lost in the presence of detectable antibodies to hFVIII (Bethesda unit >1) following vector administration. The immunosuppression regimen was performed with rituximab (250 mg/m², IV at 4 weekly intervals, total of 4 infusions) and cyclophosphamide (300 mg/m², slow intravenous infusions every 15 days, total of 8 doses over 4 months) as described previously (Mcintosh *et al.* (2013)
25 Therapeutic levels of FVIII following a single peripheral vein administration of rAAV vector encoding a novel human factor VIII variant. Blood, 121, 3335-44.).

6.3.8 Vector biodistribution

Tissue samples (inguinal lymph nodes, lumbar lymph nodes, muscle [right gastrocnemius], right testis, pancreas, right kidney, spleen, right lung, heart, and liver)
30 from C57BL/6J mice were snap frozen at the time of necropsy, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA). Detection and

quantification of vector genomes copies (GC) in extracted DNA and relative hFVIII transcript expression in extracted RNA were performed by real-time PCR as described previously. Briefly, vector GCs and RNA were quantified using primers/probe designed against the hFVIII transgene sequence of the vector. Quantification of GC from liver
5 was performed on one liver sample from each mouse (n=3/group). RNA relative transcript expression was determined using the $\Delta\Delta CT$ of each sample normalized to 18S expression.

The vector biodistribution was evaluated for a subset of 18 of the AAVrh10 enhancer/promoter vectors. AAVrh10 vectors expressing hFVIIIco IV were
10 administered at a dose of 10^{11} GC per mouse into 6 to 8 week old C57BL/6J wild type mice. Mice were necropsied at day 14 post-vector administration and muscle (right gastrocnemius), right testis, pancreas, right kidney, spleen, right lung, heart, and liver was collected. DNA and RNA were extracted and vector GCs and RNA transcript levels, respectively, were quantified using primers/probe designed against the hFVIII
15 transgene sequence of the vector. There were no significant differences in liver vector GC (Fig. 20A) or RNA transcript levels (FIG. 20B) across the vector administered groups, and there were no detectable GC or RNA in the control (PBS) administered groups. However, there was a trend towards higher RNA transcript levels in the liver for vectors that used the A1AT promoter for expression, regardless of the enhancer
20 sequences (FIG. 20B). For the other tissues collected, hFVIII RNA transcript levels were on average 1000-fold lower than in the liver but high extrahepatic expression was seen in muscle and heart in the C57BL/6J mice following administration of E01.TBG-S1, E02.A1AT, E09.A1AT, and E09.TTR (FIG. 21).

6.4. Testing of Vector

25 Characterization assays including serotype identity, empty particle content and transgene product identity are performed. Descriptions of all the assays appear below.

6.4.1 Genomic Copy (GC) Titer

An optimized quantitative PCR (oqPCR) assay is used to determine genomic copy titer by comparison with a cognate plasmid standard. The oqPCR assay utilizes
30 sequential digestion with DNase I and Proteinase K, followed by qPCR analysis to measure encapsidated vector genomic copies. DNA detection is accomplished using

sequence specific primers targeting the PA75 polyA region in combination with a fluorescently tagged probe hybridizing to this same region. Comparison to the plasmid DNA standard curve allows titer determination without the need of any post-PCR sample manipulation. A number of standards, validation samples and controls (for background and DNA contamination) have been introduced into the assay. This assay has been qualified by establishing and defining assay parameters including sensitivity, limit of detection, range of qualification and intra and inter assay precision. An internal AAVrh.10 reference lot was established and used to perform the qualification studies.

6.4.2 Vector Capsid Identity: AAV Capsid Mass spectrometry of VP3

Confirmation of the AAV2/hu.37 or AAV2/rh.10 serotype of the vector is achieved by an assay based upon analysis of peptides of the VP3 capsid protein by mass spectrometry (MS). The method involves multi-enzyme digestion (trypsin, chymotrypsin and endoproteinase Glu-C) of the VP3 protein band excised from SDS-PAGE gels followed by characterization on a UPLC-MS/MS on a Q-Exactive Orbitrap mass spectrometer to sequence the capsid protein. A tandem mass spectrometry (MS) method was developed that allows for identification of certain contaminant proteins and deriving peptide sequence from mass spectra.

6.4.3 Empty to Full Particle Ratio

Vector particle profiles are using analytical ultracentrifugation (AUC) Sedimentation velocity as measured in an analytical ultracentrifuge is an excellent method for obtaining information about macromolecular structure heterogeneity, difference in conformation and the state of association or aggregation. Sample was loaded into cells and sedimented at 12000 RPM in a Beckman Coulter Proteomelab XL-I analytical ultracentrifuge. Refractive index scans were recorded every two minutes for 3.3 hours. Data are analyzed by a c(s) model (Sedfit program) and calculated sedimentation coefficients plotted versus normalized c(s) values. A major peak representing the monomeric vector should be observed. The appearance of peaks migrating slower than the major monomeric peak indicate empty/misassembled particles. The sedimentation coefficient of the empty particle peak is established using empty AAV8 particle preparations. Direct quantitation of the major monomeric peak and preceding peaks allow for the determination of the empty to full particle ratio.

6.4.4 Infectious Titer

The infectious unit (IU) assay is used to determine the productive uptake and replication of vector in RC32 cells (rep2 expressing HeLa cells). Briefly, RC32 cell in 96 well plates are co-infected by serial dilutions of vector and a uniform dilution of Ad5 with 12 replicates at each dilution of rAAV. Seventy-two hours after infection the cells are lysed, and qPCR performed to detect rAAV vector amplification over input. An end-point dilution TCID₅₀ calculation (Spearman-Kärber) is performed to determine a replicative titer expressed as IU/ml. Since “infectivity” values are dependent on particles coming into contact with cells, receptor binding, internalization, transport to the nucleus and genome replication, they are influenced by assay geometry and the presence of appropriate receptors and post-binding pathways in the cell line used. Receptors and post-binding pathways critical for AAV vector import are usually maintained in immortalized cell lines and thus infectivity assay titers are not an absolute measure of the number of “infectious” particles present. However, the ratio of encapsidated GC to “infectious units” (described as GC/IU ratio) can be used as a measure of product consistency from lot to lot.

7. Example 2: Protocol for Treating Human Subjects

This Example relates to a gene therapy treatment for patients with genetically confirmed X-linked hemophilia A due to mutations in the clotting factor 8 (FVIII) gene. In this example, the gene therapy vector, AAVhu.37.hFVIII, a replication deficient adeno-associated viral vector hu.37 (AAVhu.37) expressing hFVIII is administered to patients with hemophilia A. Efficacy of treatment can be assessed using FVIII levels as a surrogate for transgene expression. Primary efficacy assessments include FVIII levels at about 12 weeks post treatment, with persistence of effect followed thereafter for at least 1 year. Long term safety and persistence of transgene expression may be measured post-treatment in liver biopsy samples.

7.1. Gene Therapy Vector - AAV.hFVIII

7.1.1. AAVhu.37.hFVIII

The AAVhu.37.hFVIII vector consists of the AAV vector active ingredient and a formulation buffer. The external AAV vector component is a serotype hu.37, $T = 1$ icosahedral capsid consisting of 60 copies of three AAV viral proteins, VP1,

VP2, and VP3, at a predicted ratio of 1:1:10. The capsid contains a single-stranded DNA recombinant AAV (rAAV) vector genome (FIG. 1-FIG. 4).

The genome contains a human factor VIII (FVIII) transgene flanked by the two AAV inverted terminal repeats (ITRs). An enhancer, promoter, human factor VIII (hFVIII) coding sequence, and polyadenylation (polyA) signal comprise a B domain
5 deleted, codon optimized human FVIII transgene. The ITRs are the genetic elements responsible for the replication and packaging of the genome during vector production and are the only viral *cis* elements required to generate rAAV. In one embodiment, expression of the human FVIII coding sequence is driven from the transthyretin
10 promoter (SEQ ID NO: 7). In another embodiment, expression of the human FVIII coding sequence is driven from the modified A1AT promoter (SEQ ID NO: 9). The construct includes at least one enhancer element to stimulate promoter activity. In one embodiment, an enTTR enhancer (SEQ ID NO: 5) is included. In another embodiment, two copies of the ABP-S enhancer (SEQ ID NO: 6) proceed one copy of the enTTR
15 enhancer (SEQ ID NO: 5). A synthetic polyA signal of about 75 nt (SEQ ID NO: 10) is included to mediate termination of human FVIII mRNA transcripts.

The vector is supplied as a suspension of AAVhu.37.hFVIII vector in formulation buffer. In one embodiment, the formulation buffer is 0.001% Pluronic F-68 in TMN200 (200 mM sodium chloride, 1 mM magnesium chloride, 20 mM Tris, pH 8.0).

20 Details of the vector manufacturing and characterization of the vectors, are described in the sections below.

7.1.2. AAVrh.10.hFVIII

The AAVrh.10.hFVIII vector consists of the AAV vector active ingredient and a formulation buffer. The external AAV vector component is a serotype
25 rh.10, $T = 1$ icosahedral capsid consisting of 60 copies of three AAV viral proteins, VP1, VP2, and VP3, at a predicted ratio of 1:1:10. The capsid contains a single-stranded DNA recombinant AAV (rAAV) vector genome (FIG. 1-FIG. 4).

The genome contains a human factor VIII (FVIII) transgene flanked by the two AAV inverted terminal repeats (ITRs). An enhancer, promoter, human factor
30 VIII (hFVIII) coding sequence, and polyadenylation (polyA) signal comprise a B domain deleted, codon optimized human FVIII transgene. The ITRs are the genetic elements

responsible for the replication and packaging of the genome during vector production and are the only viral *cis* elements required to generate rAAV. In one embodiment, expression of the human FVIII coding sequence is driven from the transthyretin promoter (SEQ ID NO: 7). In another embodiment, expression of the human FVIII coding sequence is driven from the modified A1AT promoter (SEQ ID NO: 9). The construct includes at least one enhancer element to stimulate promoter activity. In one embodiment, an enTTR enhancer (SEQ ID NO: 5) is included. In another embodiment, two copies of the ABP-S enhancer (SEQ ID NO: 6) precede one copy of the enTTR enhancer (SEQ ID NO: 5). A synthetic polyA signal of about 75 nt (SEQ ID NO: 10) is included to mediate termination of human FVIII mRNA transcripts.

The vector is supplied as a suspension of AAVrh.10.hFVIII vector in formulation buffer. In one embodiment, the formulation buffer is 0.001% Pluronic F-68 in TMN200 (200 mM sodium chloride, 1 mM magnesium chloride, 20 mM Tris, pH 8.0).

7.2. Patient Population

Severe hemophilia A patients are the chosen study population for several reasons. Severe hemophilia A patients are defined as having less than 1% of normal Factor VIII (FVIII) activity thus requiring frequent infusions of FVIII to control their bleeding diathesis. This represents a significant burden with respect to carrying on a normal life and in addition, the blood levels of FVIII go through the well-known peaks and troughs pattern, which is not optimal. The fact that FVIII blood levels in severe patients is less than 1% makes it possible to reliably measure even low to moderate increases in FVIII blood levels after AAV.hFVIII has been administered. Recent clinical trials have borne out the validity of this approach.

Patients who are candidates for treatment are preferably adult males ≥ 18 years of age, diagnosed with moderate/severe or severe hemophilia A. In one embodiment, the patient has a baseline FVIII activity $\leq 2\%$ of normal or documented history of FVIII activity $\leq 2\%$. In some embodiments, a patient < 18 years of age can be treated. Candidates for treatment include subjects who have had at least 3 bleeding episodes per year that require on-demand treatment with FVIII. Other candidates for treatment include subjects who are treated with a prophylactic regimen of FVIII. Other criteria demonstrating that the subject is appropriate for treatment includes at least 100 days

exposure history to FVIII; no documented history of inhibitors (neutralizing antibodies) to exogenous FVIII; no known allergic reaction to exogenous FIX or any component of AAV.hFVIII.

Patients that are treated can have a baseline serum AAVhu.37 or AAVrh.10 (as appropriate for the chosen vector) neutralizing antibody (Nab) titer $\leq 1:5$.

Subjects may be permitted to continue their standard of care treatment(s) (e.g., replacment FVIII) prior to and concurrently with the gene therapy treatment at the discretion of their caring physician. In the alternative, the physician may prefer to stop standard of care therapies prior to administering the gene therapy treatment and, optionally, resume standard of care treatments as a co-therapy after administration of the gene therapy.

7.3. Dosing & Route of Administration

Patients receive a single dose of AAVrh.10.hFVIII or AAVhu.37.hFVIII administered via a peripheral vein by infusion. The dose of AAVrh.10.hFVIII or AAVhu.37.hFVIII administered to a patient is about 5×10^{11} GC/kg or 1.6×10^{12} GC/kg or 5×10^{12} GC/kg or 1×10^{13} GC/kg. In order to ensure that empty capsids are removed from the dose of AAVrh.10.hFVIII or AAVhu.37.hFVIII that is administered to patients, empty capsids are separated from vector particles by cesium chloride gradient ultracentrifugation or by ion exchange chromatography during the vector purification process, as discussed above.

7.4. Measuring Clinical Objectives

Primary assessments are for safety of the administered product. The following assessments are conducted twice a week for 4 weeks after the administration of the product, weekly from week 6 to week 12, monthly throughout the remainder of the first year and at 6 month intervals for a total period of 5 years.

- a. Physical examination
- b. ECG
- c. Biochemical assessment: Serum electrolytes, BUN, creatinine, calcium, phosphate, total protein, albumin, LDH, CPK, AST, ALT, alkaline phosphatase, bilirubin
- d. Hematological assessment: CBC and differential, coagulation profile
- e. Urinalysis

f. Immunological assessment:

g. Serological response to hu.37 or rh.10 capsid and to Factor VIII

h. T cell response to hu.37 or rh.10 capsid and Factor VIII antigens

i. Assessment of vector DNA; qPCR measurements in plasma, urine and saliva

5 Secondary assessments are based on measurements of transgene expression and activity as determined by

a. Plasma Factor VIII levels and Factor VIII activity

b. Clinical assessment of replacement Factor VIII requirements and frequency of spontaneous bleeding episodes

10

8. Example 3: Manufacture of AAV.hFVIII

8.1. Plasmids used to Produce AAV.hFVIII

AAVrh.10.hFVIII is produced by 3 plasmid DNA transfection of human HEK 293 MCB cells with:

15

(i) a vector plasmid as described in Section 8.2.1.1-8.2.1.4

(ii) an AAV helper plasmid termed pAAV2.rh10.KanR containing the AAV rep2 and cap rh10 wild-type genes described in Section 8.2.2.1 and

(iii) a helper adenovirus plasmid termed pAdDeltaF6(Kan) described in Section 8.2.3

20

AAVhu.37.hFVIII is produced by 3 plasmid DNA transfection of human HEK 293 MCB cells with:

(i) a vector plasmid as described in Section 8.2.1.1-8.2.1.4

25 (ii) an AAV helper plasmid termed pAAV2.hu.37.KanR containing the AAV rep2 and cap hu.37 wild-type genes described in Section 8.2.2.2 and

(iii) a helper adenovirus plasmid termed pAdDeltaF6(Kan) described in Section 8.2.3

8.2.1 Cis plasmids (vector genome expression construct):

30

8.2.1.1 pAAV.E03.p3.hF8co-SQ.PA75 containing the human FVIII expression cassette (FIG. 1). This *cis* plasmid encodes the rAAV vector genome.

Expression of the human FVIII-SQco cDNA is driven from the TTR promoter with an enTTR enhancer. The polyA signal for the expression cassette is an artificial polyA sequence of about 75nt.

Description of the Sequence Elements

- 5 1. *Inverted terminal repeats (ITR)*: AAV ITRs are sequences that are identical on both ends, but found in opposite orientation. The AAV2 (GenBank # NC001401) ITR sequences function as both the origin of vector DNA replication and the packaging signal for the vector genome, when AAV and adenovirus (ad) helper functions are provided in *trans*. As such, the ITR sequences represent the only *cis*
10 acting sequences required for vector genome replication and packaging. The 5' ITR sequence used in the exemplified vector is shown in SEQ ID NO: 11. The 3' ITR sequence used in the exemplified vector is shown in SEQ ID NO: 12.
2. *TTR promoter*: The transthyretin promoter (SEQ ID NO: 7) and is used to drive high-level, liver specific hFVIII gene expression.
- 15 3. *TTR Enhancer (enTTR)*: A 100 bp enhancer sequence (SEQ ID NO: 5) from transthyretin is present in the vector expression cassette to increase expression of FVIII.
4. *Human coagulation factor VIII (FVIII) cDNA* (SEQ ID NO: 1 shows native sequence; SEQ ID NO: 2 shows codon optimized sequence). The human
20 coagulation factor 8 (FVIII) cDNA encodes a coagulation factor essential for the formation of blood clots. The hFVIII is a B-Domain Deleted sequence in which the B domain has been replaced with a short "SQ" sequence, as described herein. The hFVIII cDNA is codon optimized for expression in humans.
5. *Artificial polyadenylation signal*: (SEQ ID NO: 10) A 75bp
25 artificial polyadenylation signal provides *cis* sequences for efficient polyadenylation of the hFVIII mRNA. This element functions as a signal for transcriptional termination, a specific cleavage event at the 3' end of the nascent transcript followed by addition of a polyadenyl tail.

30 **8.2.1.2** pAAV.E12.p3.hF8co-SQ.PA75 containing the human FVIII expression cassette (FIG. 2). This *cis* plasmid encodes the rAAV vector genome.

Expression of the human FVIII-SQco cDNA is driven from the TTR promoter with a ABPS and enTTR enhancer. The polyA signal for the expression cassette is an artificial polyA sequence of about 75nt.

Description of the Sequence Elements

- 5 1. *Inverted terminal repeats (ITR)*: Same as for 8.2.1.1
2. *TTR promoter*: Same as for 8.2.1.1
3. *Enhancer*: A shortened version of the 100 bp distal enhancer from the α 1-microglobulin/bikunin precursor [ABP] to 42 bp (SEQ ID NO: 6) with two copies of the 100 bp enhancer sequence from transthyretin (enTTR) (SEQ ID NO: 5)
- 10 are present in the vector expression cassette to increase expression of FVIII.
4. *Human coagulation factor VIII (FVIII) cDNA*: Same as for 8.2.1.1
5. *Artificial polyadenylation signal*: Same as for 8.2.1.1

- 15 **8.2.1.3** pAAV.E03.p2.hF8co-SQ.PA75 containing the human FVIII expression cassette (FIG. 3). This *cis* plasmid encodes the rAAV vector genome. Expression of the human FVIII-SQco cDNA is driven from a modified A1AT promoter with a enTTR enhancer. The polyA signal for the expression cassette is an artificial polyA sequence of about 75nt.

Description of the Sequence Elements

- 20 1. *Inverted terminal repeats (ITR)*: Same as for 8.2.1.1
2. *A1AT promoter*: A modified SERINA1 [α 1-antitrypsin] promoter (SEQ ID NO: 9) and is used to drive high-level, liver specific hFVIII gene expression.
3. *TTR Enhancer (enTTR)*: A 100 bp enhancer sequence from
- 25 transthyretin is present in the vector expression cassette to increase expression of FVIII.
4. *Human coagulation factor VIII (FVIII) cDNA*: Same as for 8.2.1.1
5. *Artificial polyadenylation signal*: Same as for 8.2.1.1

- 30 **8.2.1.4** pAAV.E12.p2.hF8co-SQ.PA75 containing the human FVIII expression cassette (FIG. 4). This *cis* plasmid encodes the rAAV vector genome.

Expression of the human FVIII-SQco cDNA is driven from the TTR promoter with a ABPS and enTTR enhancer. The polyA signal for the expression cassette is an artificial polyA sequence of about 75nt.

Description of the Sequence Elements

- 5 1. *Inverted terminal repeats (ITR)*: Same as for 8.2.1.1
2. *AIAT promoter*: Same as for 8.2.1.3
3. *Enhancer*: Same as for 8.2.1.1
4. *Human coagulation factor VIII (FVIII) cDNA*: Same as for
8.2.1.1
- 10 5. *Artificial polyadenylation signal*: Same as for 8.2.1.1

8.2.2 Helper Plasmid

8.2.2.1 AAVrh10 helper plasmid pAAV2.rh10.KanR

This AAVrh10 helper plasmid (8,036 bp) encodes the 4 wild-type AAV2
15 rep proteins and the 3 wild-type AAV VP capsid proteins from serotype rh10. A novel
AAV sequence was obtained from the liver tissue DNA of a rhesus monkey and
designated AAV serotype rh10. To create the chimeric packaging construct, the AAV2
cap gene was removed from plasmid p5E18 and replaced with a PCR fragment of the
AAVrh10 cap gene amplified from a primate liver DNA to give plasmid
20 p5E18VD2/rh10. Note that the AAV p5 promoter which normally drives rep expression
is moved in this construct from the 5' end of rep to the 3' end of the rh10 cap gene. This
arrangement serves to introduce a spacer between the promoter and the rep gene (i.e., the
plasmid backbone) to down-regulate expression of rep and increase the ability to support
high titer vector production. The plasmid backbone in p5E18 is from pBluescript KS. All
25 component parts of the plasmid have been verified by direct sequencing. Finally the
ampicillin resistance gene was replaced by the kanamycin resistance gene to give
pAAV2/rh10 (Kan).

8.2.2.2 AAVhu.37 helper plasmid pAAV2.hu.37.KanR

This AAVhu.37 helper plasmid (8,036 bp) encodes the 4 wild-type AAV2
30 rep proteins and the 3 wild-type AAV VP capsid proteins from serotype hu.37. A
schematic of the pAAV2.rh10.KanR plasmid is shown below. To create the chimeric

packaging construct, the AAV2 cap gene was removed from plasmid p5E18 and replaced with a PCR fragment of the AAVhu.37 cap gene amplified from a primate liver DNA to give plasmid p5E18VD2/hu.37. The plasmid backbone in p5E18 is from pBluescript KS. All component parts of the plasmid have been verified by direct sequencing. Finally the
5 ampicillin resistance gene was replaced by the kanamycin resistance gene to give pAAV2/hu.37 (Kan).

8.2.3 pAdDeltaF6(Kan) adenovirus helper plasmid

Plasmid pAdDeltaF6(Kan) is 15,774 bp in size. The plasmid contains the regions of adenovirus genome that are important for AAV replication, namely E2A, E4,
10 and VA RNA (the adenovirus E1 functions are provided by the 293 cells), but does not contain other adenovirus replication or structural genes. The plasmid does not contain the *cis* elements critical for replication such as the adenoviral inverted terminal repeats and therefore, no infectious adenovirus is expected to be generated. It was derived from an E1, E3 deleted molecular clone of Ad5 (pBHG10, a pBR322 based plasmid). Deletions
15 were introduced in the Ad5 DNA to remove expression of unnecessary adenovirus genes and reduce the amount of adenovirus DNA from 32 kb to ~12 kb. Finally the ampicillin resistance gene was replaced by the kanamycin resistance gene to give pAdΔF6(kan). The identity of these 3 adenovirus genes were confirmed by DNA plasmid sequencing performed by Qiagen Genomic Services on the plasmid source stock that was sent to
20 Aldevron Inc. for plasmid DNA manufacturing. DNA Analysis revealed 100% homology with the 3 Adenovirus type 5 gene regions (GenBank Accession number AF369965).

8.2.4 Bacterial Master Cell Banks (MCB)

Bacterial MCBs for the three DNA production plasmids that are used to
25 support the manufacture of DTX101 were produced by Aldevron Inc. Cell banks were made from the expansion of selected cultures and extensive testing was performed for qualification of each bacterial MCB following Aldevron SOPs and in accordance with CBER recommendations. Information regarding the specifics of bacterial MCB generation and testing for each of the three plasmids are performed and recorded.

8.2.5 Plasmid DNA Manufacturing

30

All plasmids used in the production process were produced by Aldevron Inc. under its GMP-S™ quality system and infrastructure utilizing the most salient features of cGMP manufacturing; traceability, document control, and materials segregation. Information regarding the specifics of plasmid DNA generation and testing for each plasmid are performed and recorded.

8.2.6 Human Embryonic Kidney (HEK) 293 Master Cell Bank (MCB)

HEK 293 cells were originally generated by transforming HEK cells with sheared adenovirus type 5 DNA by Frank Graham and colleagues. The cells express the E1a and E1b gene products required for high-titer rAAV production. HEK293 cells are adherent and highly transfectable yielding high-titers of rAAV upon DNA plasmid transfection.

8.3 Recombinant AAV Vector Manufacturing

8.3.1 Description of the Manufacturing Process

1. Cell Seeding: A qualified human embryonic kidney 293 cell line is used for the production process. Cells are cultivated in medium composed of Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% gamma irradiated Fetal Bovine Serum (FBS). The cells are anchorage dependent and cell disassociation is accomplished using TrypLE Select, a non-animal cell dissociation reagent. The cells are maintained at 37°C (+/- 1°C), in 5% (+/- 0.5%) CO₂ atmosphere.

2. Transient Transfection: Following 3 days of growth (DMEM media + 10% FBS), Hyperstack cell culture media is replaced with fresh, serum free DMEM media and transfected with the 3 production plasmids using an optimized PEI precipitation method.

Sufficient DNA plasmid transfection complex is prepared in the BSC to transfect twenty Corning 36-layer HyperStacks (per BDS lot). Initially a DNA/PEI mixture is prepared containing 3.0 mg of pDTX.hFIX.101 vector plasmid, 60 mg of pAdDeltaF6(Kan), 30 mg of pAAV2.rh10.KanR AAV helper plasmid and GMP grade PEI (PEIPro, PolyPlus Transfection SA). After mixing well, the solution is allowed to sit at room temperature for 25 min. and then added to serum-free media to quench the reaction and then added to the Corning 36-layer Hyperstacks. The transfection mixture is

equalized between all 36 layers of the Hyperstack and the cells are incubated at 37°C (+/- 2°C) in a 5% (+/- 0.5%) CO₂ atmosphere for 5 days.

3. Cell Media Harvesting: Transfected cells and media are harvested from each Hypertack using disposable bioprocess bags by aseptically draining the medium out of the units. Following the harvest of media, the ~ 80 liter volume is supplemented with MgCl₂ to a final concentration of 2 mM (co-factor for Benzonase) and Benzonase nuclease (Cat#: 1.016797.0001, Merck Group) added to a final concentration of 25 units/ml. The product (in a disposable bioprocess bag) is incubated at 37°C for 2-3 hr in an incubator to provide sufficient time for enzymatic digestion of residual cellular and plasmid DNA present in the harvest as a result of the transfection procedure. This step is performed to minimize the amount of residual DNA in the final vector DP. After the incubation period, NaCl is added to a final concentration of 500 mM to aid in the recovery of the product during filtration and downstream tangential flow filtration.

4. Clarification: Cells and cellular debris are removed from the product using a depth filter capsule (1.2 µm/0.22 µm) connected in series as a sterile, closed tubing and bag set that is driven by a peristaltic pump. The media is passed through a Sartorius Sartoguard PES capsule filter (1.2 µm /0.22 µm) (Sartorius Stedim Biotech Inc.).

5. Large-scale Tangential Flow Filtration: Volume reduction (10-20 fold) of the clarified product is achieved using Tangential Flow Filtration (TFF) using a custom sterile, closed bioprocessing tubing, bag and membrane set produced by Spectrum Labs.

8.4 Readministration with second vector

8.4.1 Readministration of AAV3B or AAV5

The efficiency of vector readministration using AAV3B or AAV5 in rhesus macaques previously treated with AAVrh10 or AAV8 vectors was evaluated. Vectors as shown in Table 4 were produced as previously described in which the vector was recovered from the supernatant following triple transfection in HEK293 cells and purified on an iodixanol gradient. Vector titer was determined by a digital PCR method.

Twenty four male rhesus macaques (3-5 years old) were enrolled into study in 8 groups (n=3/group; Table 1) based on the status of pre-existing NAb. Macaques were injected on day zero with 1.0×10^{13} GC/kg AAV.TBG.hCG.WPRE, with the AAV vector as shown in Table 4. At week 12, macaques received a second injection with 1.0×10^{13} GC/kg AAV.TBG.hCG.WPRE, with the AAV vector as shown in Table 4. Liver biopsies were performed at week 2 and week 14, and a necropsy was performed at week 26.

Table 4: Cohort and Vector Summary

Cohort	Animal ID	1st Injection	2nd Injection
G1A	RA0931	PBS	AAV3B.TBG.rhAFP
	RA1388		
	RQ9745		
G1B	RA0923	PBS	AAV5.TBG.rhAFP
	RA1275		
	RQ9383		
G2A	RA0985	AAVrh10.TBG.rhCG.WPRE	AAV3B.TBG.rhAFP
	RQ9638		
	RQ9746		
G2B	RA0992	AAVrh10.TBG.rhCG.WPRE	AAV5.TBG.rhAFP
	RA1322		
	RA1417		
G3A	RA1234	AAV8.TBG.rhCG.WPRE	AAV3B.TBG.rhAFP
	RQ9737		
	RQ9751		
G3B	RA1339	AAV8.TBG.rhCG.WPRE	AAV5.TBG.rhAFP
	RA1390		
	RQ9805		
G4	RA0548	AAV3B.TBG.rhCG.WPRE	N/A
	RA0658		
	RQ9840		
G5	RA0968	AAV5.TBG.rhCG.WPRE	N/A
	RA1208		
	RA1239		

Expression levels of transgenes (rhCG – rhesus chorionic gonadotropin b subunit; rhAFP – rhesus alpha fetoprotein) in the serum were measured by enzyme-linked immunosorbent assay (ELISA). To measure vector DNA copies in liver, QPCR assays were performed on total cellular DNA extracted from liver samples collected

during liver biopsy and necropsy. AAV NAb assay was performed as previously described. Liver sections were stained with an anti-CG antibody for imaging.

FIG 15 shows a comparison of rhCG expression levels by AAVrh10, AAV8, AAV3B and AAV5 vectors (first vector injection). FIG 16A-16D shows rhCG
5 vector DNA copies in liver at different time points. FIG 17A-17B shows rhAFP levels after readministration (second vector injection) with AAV3B (FIG. 17A) or AAV5 (FIG. 17B) vectors expressing rhAFP. FIG 18A and FIG. 18B shows rhAFP vector genome copies in liver. FIG 19 shows differential AAV Nab response in macaques.

In naïve animals, clade E vectors (AAVrh10 & AAV8) demonstrated the
10 highest levels of periportal gene transfer with AAV5 vectors having the lowest; the periportal zone is nearest to the entering vascular supply, receives the most oxygenated blood, and is an important region of the liver for metabolic processes. AAVrh10 and AAV5 elicited higher levels of neutralizing antibodies (NAb) than AAV8 and AAV3B. Significant animal-to-animal variation in transgene expression was noted with AAV3B
15 in seronegative animals. Within the short time frame tested, NAb elicited from AAVrh10 appears to have inhibited subsequent in vivo transduction with the serologically distinct AAV3B serotype; prior exposure to AAV8 did not interfere with AAV3B transduction.

All publications cited in this specification, as well as US provisional patent
20 application numbers 62/323,336, 62/331,807, and 62/428,866, are incorporated herein by reference. Similarly, the SEQ ID NOs which are referenced herein and which appear in the appended Sequence Listing, and the sequence listing itself, are incorporated by reference. While the invention has been described with reference to particular
25 embodiments, it will be appreciated that modifications can be made without departing from the spirit of the invention. Such modifications are intended to fall within the scope of the appended claims.

(Sequence Listing Free Text)

The following information is provided for sequences containing free text under
numeric identifier <223>.

30

SEQ ID NO: (containing free text)	Free text under <223>
-----------------------------------	-----------------------

4	<223> constructed sequence
5	<223> constructed sequence
6	<223> constructed sequence
7	<223> constructed sequence
8	<223> constructed sequence
9	<223> constructed sequence
10	<223> constructed sequence
11	<223> constructed sequence
12	<223> constructed sequence
13	<223> constructed sequence
14	<223> constructed sequence
15	<223> constructed sequence
16	<223> constructed sequence
17	<223> AAVhu.37 capsid
18	<223> AAVrh.10 capsid
19	<223> constructed sequence
20	<223> AAV3B capsid

CLAIMS:

1. A recombinant adeno-associated virus (rAAV) useful as a liver-directed therapeutic for hemophilia A, said rAAV comprising an AAV capsid, and a vector genome packaged therein, said vector genome comprising:

- (a) an AAV 5' inverted terminal repeat (ITR) sequence;
- (b) a transthyretin enhancer (enTTR);
- (c) a transthyretin (TTR) promoter;
- (d) a coding sequence encoding a human Factor VIII having coagulation function;
- (e) an AAV 3' ITR.

2. The rAAV according to claim 1, wherein the human Factor VIII is a B-domain deleted factor VIII SQ which is about 1457 amino acid residues in length.

3. The rAAV according to claim 1 or claim 2, wherein the coding sequence of (d) is selected from SEQ ID NO: 1 and SEQ ID NO: 2.

4. The rAAV according to any of claims 1 to 3, wherein the rAAV capsid is a hu37 capsid.

5. The rAAV according to any of claims 1 to 4, wherein the AAV 5' ITR and/or AAV3' ITR is from AAV2.

6. The rAAV according to any of claims 1 to 5, wherein the vector genome further comprises a polyA which is about 75 bp in size.

7. The rAAV according to any of claims 1 to 6, wherein the vector genome is about 5 kilobases to about 5.5 kilobases in size.

8. An aqueous suspension suitable for administration to a hemophilia A patient, said suspension comprising an aqueous suspending liquid and about 1×10^{12} GC/mL to about 1×10^{14} GC/mL of a recombinant adeno-associated virus (rAAV) useful as a liver-directed therapeutic for hemophilia A, said rAAV having an AAV capsid, and having packaged therein a vector genome comprising:

- (a) an AAV 5' inverted terminal repeat (ITR) sequence;
- (b) a transthyretin enhancer (enTTR);
- (c) a transthyretin (TTR) promoter;
- (d) a coding sequence encoding a human Factor VIII having coagulation function;
- (e) an AAV 3' ITR.

9. The suspension according to claim 8, wherein the suspension is suitable for intravenous injection.

10. The suspension according to claim 8 or claim 9, wherein the suspension further comprises a surfactant, preservative, and/or buffer dissolved in the aqueous suspending liquid.

11. A method of treating a patient having hemophilia A with an rAAV according to claim 1, wherein the rAAV is delivered about 1×10^{12} to about 1×10^{14} genome copies (GC)/kg in an aqueous suspension, wherein the GC are calculated as determined based on oqPCR.

12. The method according to claim 11, wherein the rAAV is readministered at a later time point.

13. The rAAV according to claim 1, wherein the vector genome comprises nt 1–5110 of SEQ ID NO: 13.

14. The rAAV according to claim 13, wherein the rAAV capsid is an hu37 capsid.
15. The rAAV according to claim 1, wherein
the enTTR is SEQ ID NO:5;
the TTR promoter is SEQ ID NO:7;
the coding sequence is SED ID NO:2.
16. The rAAV according to claim 15 wherein the AAV 5' ITR is SEQ ID NO:11 and the AAV 3' ITR is SEQ ID NO:12.
17. The rAAV according to either of claims 15 or 16, further comprising a polyA sequence of SEQ ID NO:10.
18. The rAAV according to any one of claims 15–17, wherein the rAAV capsid is an hu37 capsid.