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# Liver-Directed Adeno-Associated Viral Gene Therapy for Hemophilia

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**Abstract**

Hemophilia A and B are monogenic bleeding disorders resulting from loss of functional coagulation factors VIII or IX, respectively. Prophylactic treatment requires frequent intravenous injections of exogenous factor VIII (F.VIII) or factor IX (F.IX), due to the short half-life of both factors. Hemophilia patients are at risk of developing neutralizing antibodies to F.VIII (~25-30%) or F.IX (~2-4%), which require the use of expensive bypass agents and immune tolerance induction protocols. Viral vector mediated liver gene transfer of F.VIII or F.IX offers an alternative treatment for hemophilia with easily defined clinical endpoints and no need for strict regulation of coagulation factor expression, as both proteins circulate as inactive zymogens. Adeno-associated viral (AAV) vectors are derived from a non-pathogenic human virus that efficiently transduce non-dividing cells, such as hepatocytes, and provide stable transgene expression. *In vivo* liver gene transfer of AAV-F.VIII and -F.IX vectors has restored hemostasis in murine and canine hemophilia models long-term, and has also been shown to induce immune tolerance. Consequently, two Phase I/II clinical trials have been conducted, based on hepatic AAV-FIX gene transfer to patients with severe hemophilia B. The first trial, utilizing serotype 2, demonstrated transient correction, which was limited by a cellular immune response against the viral capsid. However, sustained therapeutic expression has been achieved in a second trial, using AAV8 for expression of a codon-optimized F.IX transgene. Translation of F.VIII gene transfer studies into the clinic may require additional optimization of gene transfer and vector to effectively express the larger cDNA of F.VIII.

**Keywords:** Hemophilia; Adeno-associated virus; AAV; Liver; Factor VIII; Factor IX; Gene transfer

## Hemophilia

Hemophilia is a bleeding disorder resulting from the loss of functional coagulation factors VIII (Hemophilia A) and IX (Hemophilia B). The severity and frequency of bleeding episodes is related to residual coagulation factor activity, patients with less than 1% activity often suffer from spontaneous bleeds. Bleeding episodes are treated in the clinic with intravascular (IV) administration of exogenous Factor VIII or IX from plasma concentrates or recombinant protein. Clinical data from treated patients show that maintaining coagulation factor levels above 1% has a significant impact on the frequency of spontaneous bleeding and above 5% results in the elimination of spontaneous bleeds. Unfortunately, a subset of patients (hemophilia A ~25-30%, hemophilia B ~2-4%) is at risk for developing neutralizing antibodies to the exogenously administered coagulation factors. These inhibitors abolish therapeutic benefit and require expensive long-term immune tolerance induction (ITI) protocols for the eradication of inhibitors. Prophylactic treatment of hemophilia patients requires frequent IV injections to maintain steady state levels of the respective coagulation factor, which presents a burden on the patient and is quite expensive. Novel factor products with longer half-life in circulation are currently being developed. Since the liver is the natural site of FVIII and F.IX synthesis, liver gene transfer of a functional copy of F.VIII or F.IX offers an alternative treatment approach with the potential to provide stable therapeutic FVIII or F.IX expression from a single injection. Adeno-associated virus (AAV) vectors have shown a promising platform for long-term liver gene transfer.

## Adeno-Associated Virus (AAV)

AAV is a non-pathogenic single stranded DNA parvovirus with a genome size of approximately 4.7kb. Serotypes with distinct tissue tropisms have been isolated from multiple vertebrate species, including humans [1]. Viral vectors derived from AAV are devoid of viral genes and instead contain an expression cassette for the gene of interest, which is limited to ~5kb in length. Several factors make AAV vectors ideally suited for liver gene transfer including the ability to infect non-dividing cells such as hepatocytes, low immunogenicity [2-4], and the persistence of vector genomes as episomal concatamers [5,6].

The AAV2 serotype was the first serotype developed as a vector for gene transfer and has been tested extensively in both small and large animals and in two human clinical trials for muscle [7] and liver gene transfer of hF.IX [8]. Despite efficient infection of murine liver, AAV genomes detected in almost 100% of hepatocytes, only roughly 5% of murine hepatocytes expressed transgene [9-11]. Several steps in AAV2 vector infection have been identified that restrict murine liver gene transfer. Since AAV vectors have a single stranded DNA (ssDNA) genome, expression of the gene of interest requires conversion to double stranded DNA (dsDNA), this step acts as a limiting step in transgene expression [12,13]. A cellular protein, FKBP52, was shown to interact with the AAV genome, when phosphorylated, and prevent conversion to dsDNA [14]. Two distinct approaches were taken to bypass this block. The first focused on preventing the phosphorylation of the FKBP52 protein by pharmacological inhibition of phosphorylation [15] and forced expression of phosphatases TCT-PTP [16] and PP5 [17] both of which led to significant increases of *in vivo* transgene expression. Toxicity studies from over-expression of TCT-PTP or PP5 need to be evaluated in large animal models before consideration

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for human use. The second approach generated an AAV genome that was self-complementary, spontaneously forming into dsDNA ready for transgene expression, thus completely bypassing the need for second strand synthesis [18-20]. One drawback of scAAV vectors is that they reduce an already limited packaging capacity of AAV vectors in half thus excluding large transgenes, such as F.VIII. AAV2 viral particle intracellular trafficking and uncoating also impact efficient gene transfer [21,22]. For AAV2, a large fraction of viral particles are retained in the cytoplasm upon cell entry. The capsid is phosphorylated by EGFR-PTK, which signals for ubiquitination and targeting of the viral particle to the proteasome [23]. Mutation of surfaced exposed tyrosine residues to phenylalanine, in particular residues 444, 500, and 730 resulted individually in improved gene transfer of murine liver [24] and when combined further enhanced gene expression [25]. Of the currently identified serotypes, AAV8, isolated from non-human primates [26], has the highest level of liver gene transfer in mice [27,28]. AAV8 serotype vectors have faster viral uncoating compared to AAV2 where it is believed to allow for the pairing of plus and minus strand vector genomes into stable biologically active double stranded genomes bypassing the block on second strand synthesis observed with AAV2 [26,29].

## Hepatic Gene Transfer in Animal Models of Hemophilia

Since the coding for F.IX is ~1.4 kb in length, fitting well into an AAV vector genome, and since expression of F.IX is often more efficient than that of F.VIII, the majority of pre-clinical liver gene transfer studies (and all clinical trials thus far) with AAV vectors has been conducted for hemophilia B. F.IX protein undergoes extensive post-translational modifications before being secreted and since it is normally synthesized in the liver all the cellular machinery is in place for production of mature F.IX protein. As F.IX protein circulates as an inactive zymogen, there is little need for strict regulation allowing the use of strong liver specific promoters to drive gene expression. The generation of mice deficient for murine factor IX [30-32] and two spontaneous canine hemophilia B colonies [33,34] has allowed for extensive testing of safety and efficacy of AAV liver directed F.IX gene transfer.

Snyder et al. first reported the *in vivo* delivery of an AAV2 vector expressing hF.IX from a MuLV LTR promoter/enhancer (MFG) to the liver of wild type C57BL/6 mice via the portal vein. The vector injected mice had long-term hF.IX expression (up to nine months) with no indication of liver toxicity and infiltration of immune cells [9]. Stable hF.IX expression was also obtained by Nakai et al. following portal vein delivery in C57BL/6 mice of an AAV2 vector expressing hF.IX from the EF1α promoter for a duration of six months [35]. Based on these initial studies, Snyder et al. administered AAV2 MFG-hF.IX vector to hemophilia B mice and a AAV2 MFG-cF.IX vector hemophilia B dogs via the portal vein resulting in long-term correction in both animal models [36]. In parallel, Wang et al. demonstrated long-term correction in hemophilia B mice [37] and dogs [38] using an AAV2 vector expressing canine F.IX(cF.IX) from a liver specific promoter construct. Mount et al. demonstrated sustained correction in hemophilia B dogs with a null mutation, which have a higher risk for developing inhibitors, following liver directed gene transfer of an AAV2 vector expressing cF.IX from an ApoE/hAAT liver specific promoter [39] which was shown at least 8 years following gene transfer [40].

A major concern for AAV gene transfer of hF.IX to human patients is the risk of inducing an immune response against hF.IX protein that would render patients unresponsive to exogenous F.IX protein therapy. In immune competent mice, the immune responses to hF.IX expressed from an AAV2 vector is dependent on the route of delivery and the underlying F9 mutation [41,42]. All mice receiving an intramuscular (IM) injection of an AAV2 hF.IX vector generated inhibitory antibodies to hF.IX protein, while with intravascular (IV) and portal vein deliver the majority of animals remained free from inhibitors. Based on these observations and previous examples of the tolerizing nature of liver expressed proteins [43], Mingozzi et al. designed experiments showing induction of tolerance to hF.IX in four different strains of mice using an AAV2 vector with an ubiquitous EF1α promoter or a liver-specific ApoE enhancer/$\alpha_1$-antitrypsin (ApoE/hAAT) promoter [44]. The rate of tolerance induction was shown to be associated with the levels of expressed F.IX protein. $CD4^+$ T cells from vector treated mice failed to proliferate *in vitro* following F.IX protein stimulation. Additionally, adoptive transfer of total splenocytes or $CD4^+$ lymphocytes but not CD4-depleted splenocytes from vector treated mice into naïve mice suppressed F.IX specific antibody production upon subsequent immunization, demonstrating active suppression by $CD4^+$ T cells as part of the tolerance mechanism. In addition, tolerance induction failed in Fas-deficient mice, suggesting a requirement for activation induced cell death [45]. A later study by Dobrzynski et al. found that hepatic-derived antigen expressed from an AAV vector induced several changes to the transgene product-specific $CD4^+$ T cell population, which included deletion, and induction of an anergic phenotype and enrichment for $CD4^+CD25^+$ Treg in the remaining cells [46]. More recent studies dissected the immune regulatory aspect further and found that hepatic viral gene transfer results in TGF-β dependent induction of $CD4^+CD25^+$ Treg, which are required for tolerance to the transgene product and capable of suppressing antibody and T cell responses. A regulatory response in the liver and expression of the suppressive cytokine IL-10 is required to prevent $CD8^+$ T cell responses against transduced hepatocytes [45,47-51]. These findings are further reviewed elsewhere [43,52-54].

## AAV2 Hepatic Gene Transfer Clinical Trial

Based on correction of bleeding diathesis and absence of the induction of inhibitors in both small and large models for hemophilia B by an AAV2-ApoE/hAAT-hF.IX vector and safety and persistence of an intramuscular AAV2 hF.IX clinical trial [7,55], a phase I/II clinical trial was initiated [8]. Although AAV is non-pathogenic, natural infection in humans occur with a more immunogenic helper virus, such as adenovirus, which can activate immune responses against AAV, such as the generation of neutralizing antibodies (NAB) against the viral capsid protein [56-58]. These capsid NABs have been shown to effectively block *in vivo* gene transfer to murine, nonhuman primate, and human livers at low titers [8,59-61]. A wide range of NAB titers against AAV serotypes in humans have been reported all, showing that NAB against AAV2 are the most prevalent [62-64]. Therefore, patients enrolled in the clinical trial were screened for NAB titer against AAV2 with those with a NAB titer > 1:20 being excluded from the trial.

Subjects were enrolled in three dose cohorts ($8x10^{10}$, $4x10^{11}$, and $2x10^{12}$ vg/kg) and injected in the hepatic artery with the AAV2 ApoE/hAAT hF.IX vector. In the two lower dose cohorts, circulating F.IX levels remained less than 1%, with no sign of liver toxicity in the first four treated subjects (A-D). Subject E in the highest dose cohort had transient therapeutic expression of F.IX above 11% of normal that declined to baseline levels coinciding with a self-resolving rise in liver transaminase levels. Subject F (also in the high dose cohort, with a NAB titer of 1:17 against AAV2) had no long-term increase in circulating F.IX protein and showed no signs of liver damage, underscoring the

impact of low NAB titers on efficient liver gene transfer and suggesting that the observed liver toxicity requires viral entry into hepatocytes. An additional subject G was enrolled in the mid dose cohort of $4x10^{11}$ vg/kg and had a mild transient rise in liver transaminases with F.IX levels remaining < 1%. Lymphocytes collected pre and post vector administration in subject G were used to assess T cell responses to vector capsid and F.IX protein. Surprisingly, an ELISPOT assay using peptide pools for AAV2 capsid and F.IX proteins showed expansion of AAV2 capsid-specific but not F.IX-specific CD8 T cells [8,65]. Studies conducted in control human subjects revealed the existence of capsid specific memory CD8 T cells that could be reactivated following exposure to AAV2 and other AAV serotypes, such as AAV8 [65]. Human hepatocytes infected with a similar AAV2 vector used in the clinical trial were capable of processing and presenting the capsid epitope on MHCI molecules and were lysed by capsid specific $CD8^+$ T cells [66]. Although the evidence suggested that a capsid T cell response resulted in destruction of F.IX expressing hepatocytes, no previous studies in animal models predicted this response [67]. Repeated attempts to mimic the response observed in the clinical trial in mice [68-74] and nonhuman primates (which, unlike mice, are naturally infected with AAV) have been unsuccessful [75]. In a recent study, Li et al. compared AAV capsid specific T cells found in naturally infected humans and rhesus macaques [76]. While rhesus macaques had detectable capsid specific $CD4^+$ and $CD8^+$ T cells, there were profound differences in the frequency and functionality of these capsid specific T cells compared to humans. These results may explain why the nonhuman primate model could not recapitulate the capsid immune response observed in the human clinical trial. Lack of animal studies that could reproduce the transaminitis observed in the human AAV2 F.IX trial has prompted alternative theories to explain the loss of F.IX expression. Two prominent theories are the carry over of rep/cap plasmid and the reading of an alternative reading in the F.IX cassette [77]. The former theory has been evaluated and seems unlikely to be a contributing factor [78], while the latter is also not supported by animal studies.

Based on strong evidence of a capsid $CD8^+$ T cell response being responsible for loss of F.IX expression the AAV2 F.IX clinical trial has been modified to include immune suppression (IS) prior to and following vector administration, until vector particles are degraded and cleared from hepatocytes, to prevent reactivation of $CD8^+$ T cells. Studies performed in nonhuman primates showed that transient IS does not alter AAV transduction [79,80]. But, careful selection of suppression agents is warranted. Although MMF (Mycophenolate Mofetil) and sirolimus were well tolerated, addition of a third agent daclizumab, an anti IL-2 receptor antibody, resulted in the generation of inhibitors against F.IX, likely because of depletion of Treg [80]. Along this arm, a recent study investigated the use of a clinically approved proteasome inhibitor, bortezomib, to prevent AAV2 capsid peptide presentation on MHC I molecules. Finn et al. showed a dose dependent effect of bortezomib treated hepatocytes on capsid antigen presentation following AAV2 transduction [81]. Such a therapy in combination with IS may further reduce the risk of capsid antigen presentation.

Data from the initial AAV2 F.IX liver gene transfer clinical trial suggest that vector doses $4x10^{11}$ vg/kg and lower are well tolerated with only one subject G (with the lowest pre-treatment NAB titer within the dose cohort) with a mild liver toxicity that was asymptomatic and spontaneously resolved [8]. With this target in mind, enhancing F.IX expression at least 10 fold could potentially provide long-term therapeutic F.IX levels in the absence of liver toxicity. *In vivo* delivery of bortezomib has been show to increase F.IX transgene expression in mice [73,81,82] and may have a dual function in reducing the risk of activating capsid $CD8^+$ T cells. In murine hemophilia B models the use of alternative AAV serotypes, such as AAV2 tyrosine mutant vectors [24,25] and AAV8 [83] resulted in F.IX expression levels in excess of 10 fold over AAV2. Studies are ongoing to assess if the AAV2 tyrosine mutant vectors also enhance expression in a large animal canine hemophilia B model.

## AAV8 Hepatic Gene Transfer Clinical Trial

Nathwani et al. selected an AAV vector that contained three different elements known to individually enhance liver gene expression in mice namely, an AAV8 capsid serotype [28], a scAAV genome with an optimized liver specific promoter (LP-1), and a codon optimized hF.IX gene (scAAV8 LP-1 cohF.IX). Their scAAV8 LP-1 cohF.IX vector led to substantially higher F.IX levels compared to control vectors in hemophilia B mice and nonhuman primates [84] and importantly provided similar therapeutic expression levels from peripheral vein delivery [85]. Long-term follow-up (~5 years) of nonhuman primates treated with the scAAV8 LP-1 cohF.IX vector showed sustained therapeutic expression of F.IX, absence of capsid specific $CD8^+$ T cells, and no indications of toxicity [86]. Therapeutic and safety data obtained from nonhuman primate studies prompted initiation of a second clinical trial of AAV liver gene transfer for hemophilia B using the scAAV8 LP-1 cohF.IX vector.

As with the AAV2 F.IX trial subjects with existing or previous F.IX inhibitors were excluded. Additionally, subjects with F.IX mutations, deletions, or inversions associated with increased risk of inhibitors were excluded, as well as any subjects testing positive for NABs to AAV8 based on a passive transfer assay in immune deficient mice. Subjects in the scAAV8 LP-1 cohF.IX trial were administered vector by the peripheral vein in three escalating dose cohorts ($2x10^{11}$ vg/kg, $6x10^{11}$ vg/kg, and $2x10^{12}$ vg/kg). As reported by Nathwani et al. six subjects have been treated and are stably expressing F.IX protein in the range of 2-11% normal [87]. Both subjects in the low dose cohort had detectable F.IX levels ~2% normal with no indication of an activated capsid specific T cell response. In the mid dose cohort one of two subjects (Subject 3) had pre-existing NAB to AAV8 resulting in lower F.IX expression immediately following gene transfer that later stabilized to 1-3% normal levels. The second subject had peak levels of 4% that remained stable up to 3 months and then declined to 2-3% for unknown reasons. Capsid specific CD4 and CD8 T cells were detected, with no indication of liver damage or changes in F.IX expression levels. Both subjects in the high dose cohort had peak F.IX expression of 8-12% normal, but Subject 5 suffered from a grade 3 transaminitis, with concomitant increase in capsid specific T cells, that were ablated with prednisolone treatment, resulting in a drop in F.IX levels to 3% normal. The second subject (Subject 6) in the high dose cohort had a rise in liver enzymes 62 days post gene transfer to the upper range of normal and a drop in F.IX levels to 5%. Treatment with prednisolone returned liver enzymes levels back to baseline and F.IX levels have returned to 8-12%. Both subjects in the high dose cohort had expansion of capsid specific T cells. To date there have been no indications of any immune responses against F.IX in vector treated subjects. Interestingly, similar expression levels of F.IX and transaminitis were also observed at the same dose of $2x10^{12}$ vg/kg in the AAV2 hF.IX trial [8] which suggests that this dose is a threshold for capsid antigen presentation on hepatocytes. The similar expression of F.IX obtained with both vectors may reflect the fact that the ssAAV2 hF.IX vector was delivered by hepatic artery, while the scAAV8 hF.IX vector was administered by peripheral vein (Table 1).

| ssAAV2 hF.IX | | | | | | |
|---|---|---|---|---|---|---|
| Subject | Dose vg/kg | NAB titer[1] | Peak F.IX (%) | Sustained F.IX (%) | LFT[2] | Capsid specific T cells |
| A | $8x10^{10}$ | N/A | < 1 | < 1 | WNL | ND |
| B | $8x10^{10}$ | 1:2 | < 1 | < 1 | WNL | ND |
| C | $4x10^{11}$ | 1:2 | < 1 | < 1 | WNL | ND |
| D | $4x10^{11}$ | 1:11 | < 1 | < 1 | WNL | ND |
| G | $4x10^{11}$ | < 1:2 | < 1 | < 1 | < Grade 1 | + |
| E | $2x10^{12}$ | 1:2 | 11 | < 1 | Grade 3 | ND |
| F | $2x10^{12}$ | 1:17 | 3 | < 1 | WNL | ND |
| scAAV8 hF.IX | | | | | | |
| Subject | Dose vg/kg | NAB titer relative units[3] | Peak F.IX (%) | Sustained F.IX (%) | LFT[2] | Capsid specific T cells |
| S1 | $2x10^{11}$ | 1 | 2 | 2 | WNL | - |
| S2 | $2x10^{11}$ | 12 | 2 | 2 | WNL | - |
| S3 | $6x10^{11}$ | 37 | 3 | 1-3 | WNL | + |
| S4 | $6x10^{11}$ | 1 | 4 | 2-3 | WNL | + |
| S5 | $2x10^{12}$ | 5 | 8 | 3 | Grade 3 | + |
| S6 | $2x10^{12}$ | 8 | 12 | 8-12 | WNL | + |

[1]reciprocal dilution anti AAV2 capsid titer, [2]Liver function tests (AST/ALT) [3] anti AAV8 capsid titer
WNL: within normal limits ND: not done

**Table 1:** Comparison of ssAAV2 hFIX and scAAV8 hFIX clinical trials.

The benefits of increasing to a higher vector dose will need to weighed against the risks of exacerbating an already existing capsid specific T cell response through innate immune response induction [88] and potential impacts on immune responses against the F.IX protein.

## Hepatic AAV Gene Therapy for Hemophilia A

Similar to hemophilia B, F.VIII knock-out mice were generated (by targeted deletion of exon 16 or 17) [89] and three spontaneous canine hemophilia A models are being maintained [90-94]. In two of the canine models, the underlying mutation was identified as an inversion of exons 1-22, similar to a common mutation in humans [95]. Although hemophilia A is more common than hemophilia B, progress on developing an AAV vector has been limited based on several factors including cDNA length exceeding AAV packaging capacity, poor expression and secretion of F.VIII protein, and low specific activity of human F.VIII in animal models. The F.VIII protein contains multiple domains including a large B domain that can be deleted with the resulting B domain deleted F.VIII (BDD-F.VIII) exhibiting no loss in activity as compared to full length F.VIII [96]. The BBD-F.VIII cDNA length of 4.4kb has allowed for successful packaging into AAV vectors [97,98]. Another adaption was to split the cDNA for F.VIII into two vectors expressing the heavy and light chain [99-102]. One disadvantage of this approach is that secretion of light chain (LC) is 1-2 logs higher than of heavy chain (HC). While it is possible to try and balance this by adjusting the relative LC and HC vector doses, Chen et al. described a modified HC with up to four fold improved secretion in mice [102]. Yan et al. also describe the splitting of a gene between two vectors in a processed dubbed trans-splicing that allows for expressing a cDNA larger than 5kb [103], which can also be exploited for expression of F.VIII [104]. While functional, dual vector strategies are generally less efficient as the chance for the same cell to be transduced by both vectors at an optimal ratio is low.

Burton et al. first described AAV2 dual chain hF.VIII vectors delivered via the portal vein to wild type C57BL/6 mice reaching levels of 200-400 ng/ml of biologically active hF.VIII protein [100]. Using a similar approach in hemophilia A mice, AAV2 dual chain hF.VIII or cF.VIII vectors resulted in phenotypic correction [105]. Sarkar et al. using an AAV2 vector with a synthetic albumin promoter driving BDD-murine F.VIII (mF.VIII) expression, compared different routes of delivery in hemophilia A mice and observed partial correction up to 9 months without inhibitor development [106]. Utilizing AAV8 and either a dual chain canine F.VIII or BDD canine F.VIII Sarkar et al. were able to restore 100% F.VIII activity in hemophilia A mice [101]. Mah et al. reported up to 31% of normal mF.VIII when using an AAV2 dual chain mF.VIII vector with intravenous delivery [107]. Scallan et al. reported sustained 2-4% normal levels (> 14 month) in hemophilia A dogs using intra hepatic delivery of an AAV2 TTR BDD cF.VIII vector [108]. Jiang et al. compared AAV2, AAV6, and AAV8 serotypes expressing BDD-cF.VIII (from a transthyretin-derived, TTR, promoter) in murine and canine hemophilia A models [109]. Interestingly, a large fraction of hemophilia A mice receiving high dose AAV6 and AAV8 vectors developed inhibitors to cF.VIII, while in contrast to mice, hemophilia A dogs had long-term stable expression with similar liver transduction efficiencies between the three different serotypes [109]. Sarkar et al. found a similar discordance between murine and canine gene transfer of AAV8 and AAV9 dual chain cF.VIII vectors with long-term F.VIII levels 2-2.5% of normal, and a single AAV8 treated dog with >4.5% for more than two years [110]. Ishiwata et al. showed long-term BDD cF.VIII expression in hemophilia A mice in the absence of inhibitors using an enhanced liver specific promoter [111]. Lu et al. reported complete correction in hemophilia A mice up to 8 weeks following intravenous delivery of an AAV8 CB-BDD hF.VIII vector [112]. Sabatino et al. performed a systematic study in hemophilia A dogs with peripheral vein delivery of an AAV8 BDD cF.VIII vector and portal vein delivery of AAV8 or AAV9 dual chain cF.VIII vectors [113]. Both delivery routes, serotypes, and single or dual chain F.VIII vectors led to long-term F.VIII expression, reduction in bleeding episodes, and treated dogs had no sign of long-term inhibitor formation [113].

Significant progress has been made in AAV liver gene transfer of F.VIII for treating hemophilia A in small and large animal models. What is required to translate these studies into the clinic? The major limitation from most of these studies is the use of canine F.VIII, which has been shown to not only have a higher specific activity compared to human and murine F.VIII, but is also more stable [114]. As it stands, it will be difficult to obtain regulatory approval for the delivery of a non-human gene into patients. There are several alternatives that have been shown to enhance transgene expression in ssAAV vectors including the use of tyrosine mutant capsid variants [24], the clinically approved proteasome inhibitor bortezomib for cF.VIII [82], blocking phosphorylation of FKBP52 [16,17], and the use of codon optimization which has shown to improve F.VIII expression levels in a lentiviral vector [115]. Perhaps a combination of several of the above approaches could lead to an AAV vector with suitable hF.VIII expression levels to initiate a clinical trial.

## Immunological Hurdles for AAV Liver Gene Transfer - F.VIII and F.IX Inhibitors

As most initial clinical studies will be conducted in adults with hemophilia, there is a probability that subjects will either have previously developed an inhibitor, be at risk for developing an inhibitor, or may currently have an inhibitor. Since scAAV8-cohF.IX liver gene transfer has been shown to provide long-term correction in hemophilia B subjects, what are the potential risks and benefits associated with liver gene transfer to patients with existing inhibitors? To address this, Finn et al. asked the question of whether hepatic AAV gene transfer

of cF.VIII could reverse pre-existing inhibitors in canine hemophilia A [116]. Using AAV8 dual chain cF.VIII vectors, they were able to demonstrate reversal of starting 3-8 BU inhibitors which initially generated a peak 4.5-12 BU following gene transfer and resolved to undetectable levels 4-5 weeks post vector treatment with measureable levels of circulating F.VIII protein [116]. One dog with a higher starting inhibitor of 24 BU had a peak inhibitor of 216 BU three weeks post gene transfer that declined to 0.8 BU at 80 weeks post treatment [116]. All treated animals had a sharp decline in bleeding episodes following vector treatment. Although a limited number of animals were studied and starting inhibitor levels were mild, these data open the possibility for AAV liver gene transfer for hemophilia in patients with pre-existing inhibitors or with an inhibitor prone genotype. Additionally, liver gene transfer has the potential to replace expensive long-term immune tolerance induction (ITI) protocols as a means of eradicating inhibitors in hemophilia patients.

In recent years, a murine hemophilia B model was developed that not only shows high-titer inhibitor formation against F.IX protein but also IgE formation and anaphylactic reactions, similar to observation in humans with F9 gene deletions [25,117,118] Ongoing studies will determine whether hepatic AAV-F.IX gene transfer can reverse these potentially fatal responses.

## NAB against AAV

Ideally, to correct the bleeding disorder early in life, gene transfer for hemophilia would occur in children. A retrospective study of pediatric hemophilia patients that are less than two years showed antibodies against AAV2 and AAV8 at 12 and 3% respectively [63], which is much lower than that found in the adult population [62]. One disadvantage of AAV delivery to young children is the potential loss of gene expression with the growth of the child. Primary exposure to an AAV vector in humans with low titer NAB has been shown to strongly induce a NAB response [8], which can block re-administration of same serotype. Several transient immune suppression protocols have been developed to allow re-administration of AAV vectors in mice and nonhuman primates [119-121] that may be adapted for human use. Unfortunately, there has been minimal progress made on how to eliminate existing NAB against AAV serotypes. With estimates ranging from 30 to 60% of individuals having neutralizing antibodies against AAV serotypes, how can these patients be treated with AAV liver gene transfer? Ironically, the impact of NAB against AAV may be of greater importance with the development of more efficient vectors to evade $CD8^+$ T cell activation, in that lower vector doses may be effectively neutralized at lower antibody titers. Several approaches have been considered such as plasmapheresis or pharmacological treatments that either act to lower circulating IgG titers or to transient deplete antibody secreting B cells with an anti-CD20 antibody. The risks of such procedures need to be assessed, particularly in the hemophilia population. Another approach involves modifying capsids to avoid neutralization. This has been explored with both site-directed mutagenesis of known neutralizing epitopes of AAV2 [122,123] and a directed evolution approach to select neutralizing resistant capsids [124]. While simply switching capsid serotypes may work in some cases [26,85] there appears to be higher incidence of NAB to capsids isolated from nonhuman primates, such as AAV8, in humans than previously suspected [62,63], thus limiting the effectiveness of swapping capsid. Since different capsids have different tissue tropism and gene transfer efficiencies, some capsids may not be suitable for this purpose.

## Safety Concerns for AAV Liver Gene Transfer

A major concern for gene transfer studies in humans is vertical transmission to germline cells. Studies conducted in male animal models including mice, rat, dog, and rabbit were conducted to assess the risk of germline transmission from intramuscular and intravascular delivery of an AAV2-hF.IX vector. Vector shedding into semen that was rapidly cleared with no evidence of gene transfer to germline cells was observed independent of delivery route [125,126]. An additional study in rabbits assessed the risk of a different AAV serotype, AAV8, which is currently being used in a clinical trial for hemophilia B, and showed a similar low risk of germline transmission as found with AAV2 [127]. Additionally, both human trials with AAV2 hF.IX vectors [7,8] have not shown any indication of germline transmission, validating the results obtained with the rabbit model.

Viral vector integration has been linked to insertional mutagenesis in three clinical trials of gamma retroviral vector gene transfer to human hematopoietic stem cells [128-131]. Although AAV vectors are predominantly non-integrating and remain as episomes, it has been demonstrated that integration occurs in the liver at a low frequency [132] and may pose a risk for insertional mutagenesis. A study conducted by Donsante et al. observed an increased incidence of hepatocellular carcinoma (HCC) in newborn mice given AAV liver gene transfer [133]. A follow-up study identified AAV integration sites in chromosome 12 from four liver tumors and suggested that disregulation of snoRNAs and miRNAs from vector integration resulted in the development of HCC [134]. In contrast to these findings, multiple long-term studies in mice [135,136], canines [40,113], and to date humans [8] injected with AAV vectors have no indication of increased risk of HCC from AAV insertional mutagenesis. Although animal models seem to indicate there is no risk for HCC, it would be warranted to perform long-term follow-up studies on all human trials conducted with AAV as prior experience has shown with gamma retroviral vectors and AAV lack of a response in an animal model does not necessitate lack of response in humans.

## Summary and Conclusions

Ample pre-clinical successes have been reported with AAV-F.VIII and -F.IX gene transfer vectors in murine and canine hemophilia models. AAV liver restricted gene transfer of F.VIII and F.IX results in tolerance mediated by induction of regulatory T cells. Studies in NHPs have helped to better understand potential immune responses against vector and transgene in the context of prior exposure to AAV from natural infection (both mice and dogs are not naturally infected with AAV) and have been instrumental in developing immune suppression protocols.

An initial clinical trial with AAV2 F.IX resulted in transient correction of disease in a subject at a dose of $2x10^{12}$ vg/kg. Of note, none of the treated patients developed a F.IX specific immune response, but instead two patients had an activated a memory $CD8^+$ T cell response (most likely from previous exposure to AAV from natural infection) that resulted in the elimination of transduced hepatocytes returning F.IX expression to baseline levels. First long-term correction of hemophilia B in patients has been accomplished using an scAAV8 LP-1 cohF.IX vector. Although there was indication of an activated capsid specific $CD8^+$ T cells and a mild rise in liver transaminases, rapid treatment with prednisolone resolved the transaminitis without significant loss to F.IX expression levels. It is unclear if increasing vector doses may reach a threshold for activation of an innate response and $CD8^+$ T cell capsid response and what, if any potential consequences this would entail for immune responses to vector and F.IX.

The success of the scAAV8 LP-1 cohF.IX liver gene transfer trial will hopefully not only lead to a permanent cure for hemophilia B, but pave the way for the development of AAV liver gene transfer-based therapies for hemophilia A, lysosomal storage disorders, and inherited metabolic diseases.

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