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Hemophilia B and gene therapy: a new chapter with etranacogene dezaparvovec

Xavier M. Anguela[1] and Katherine A. High[2,3]

[1]Estuary Biotherapeutics, Inc, Menlo Park, CA; [2]Rockefeller University, New York, NY; and [3]Perelman School of Medicine of the University of Pennsylvania, Philadelphia, PA

The US Food and Drug Administration (FDA)'s authorization of etranacogene dezaparvovec (Hemgenix) is a significant milestone, constituting not only the first FDA approval of a gene therapy for hemophilia but also the first approval of a liver-targeted adeno-associated virus vector gene therapy. This review summarizes the nonclinical studies and clinical development that supported regulatory clearance. Similar to other gene therapies for single gene disorders, both the short-term safety and the phenotypic improvement were unequivocal, justifying the modest-sized safety and efficacy database, which included 57 participants across the phase 2b (3 participants) and phase 3 (54 participants) studies. The most common adverse reactions included liver enzyme elevation, headache, flu-like symptoms, infusion-related reactions, creatine kinase elevation, malaise, and fatigue; these were mostly transient. One participant had hepatocellular carcinoma on a study-mandated liver ultrasound conducted 1 year after vector infusion; molecular analysis of the resected tumor showed no evidence of vector-related insertional mutagenesis as the etiology. A remarkable 96% of participants in the phase 3 trial were able to stop factor IX (FIX) prophylaxis, with the study demonstrating noninferiority to FIX prophylaxis in terms of the primary end point, annualized bleeding rate. Key secondary end points such as the annualized infusion rate, which declined by 97%, and the plasma FIX activity level at 18 months after infusion, with least squares mean increase of 34.3 percentage points compared with baseline, were both clinically and statistically significant. The FDA's landmark approval of Hemgenix as a pioneering treatment for hemophilia stands on the shoulders of >20 years of gene therapy clinical research and heralds a promising future for genomic medicines.

Introduction

The US Food and Drug Administration (FDA) approval of etranacogene dezaparvovec (Hemgenix) in November 2022 was a landmark event, both in hematology and in gene therapy. It is the first FDA approval of a gene therapy for hemophilia, the first FDA approval of a liver-directed, adeno-associated virus (AAV) vector–mediated gene therapy, and the first FDA approval of an AAV vector manufactured in insect cells. Gene therapy for hemophilia has been in clinical development for >2 decades; development of liver-directed, AAV-mediated gene therapies has occurred over the same time frame. The approval sets a precedent for multiple clinical development programs, now underway, based on AAV-mediated gene delivery to the liver, for indications ranging from plasma protein deficiencies to metabolic

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disorders to lysosomal storage disorders. The Hemgenix approval is a regular, not an accelerated approval; there are specific post-marketing requirements that address product performance in the setting of preexisting antibodies to the AAV capsid. The preclinical and clinical development was conducted primarily by uniQure (Lexington, MA), and the product is marketed by CSL Behring (King of Prussia, PA). This article will review the data that formed the basis for Hemgenix approval.

Nonclinical development program

Etranacogene dezaparvovec is the result of years of research and innovation in the field of gene therapy for hemophilia B. In the mid-2000s, it became apparent that one of the challenges of systemic AAV gene therapy was to improve hepatic transduction in order to limit the viral vector load required to achieve optimal transgene expression. Lowering vector doses was hypothesized to decrease the likelihood of eliciting anticapsid immunity and the resulting hepatocellular toxicity in the form of increased liver transaminases.[1] One of the first strategies to improve hepatic transduction was the use of alternative serotypes to AAV2 such as AAV8. AAV8 had been shown to be significantly more hepatotropic than AAV2 in murine models. The superior performance of AAV8 was associated with a higher rate of uncoating of vector genomes in the nucleus. Besides AAV8, other serotypes were also explored. In particular, AAV5 was shown to successfully transduce livers of nonhuman primates (NHPs) despite the presence of anti-AAV2 antibodies in the circulation.[2] As another modification aimed at increasing vector potency, Nathwani et al proposed that transduction efficiency could be improved by using self-complementary (sc) AAV vectors, which contained complementary dimers of a factor IX (FIX) expression cassette within individual AAV particles, thus bypassing the need for conversion of single-stranded genome to double-stranded forms in target cells.[3] The lower packaging capacity of scAAV vectors of ~2.5 kb necessitated the generation of a smaller mini-human FIX (hFIX) expression cassette including a novel liver-specific promoter termed LP1 and a codon-optimized coding sequence. This novel cassette (scAAV-LP1-hFIXco) was packaged into either AAV8 or AAV5 capsids and tested in NHPs, in which multiyear expression of FIX was observed.[4] Of note, both scAAV8-LP1-hFIXco and scAAV5-LP1-hFIXco, also known as AMT-060, reached clinical stage in academic and industry-sponsored phase 1/2 studies, respectively. Etranacogene dezaparvovec, also known as AMT-061, is a successor to AMT-060, with the same recombinant AAV5 capsid containing the identical codon-optimized gene-expression cassette, generated by a 2-nucleotide change to the wild-type (WT) human FIX sequence to encode the hyperactive, naturally occurring human[5] FIX Padua (R338L) variant.[6] The results of the trials using scAAV8-LP1-hFIXco, AMT-060, and etranacogene dezaparvovec are discussed below in "Clinical development program."

Early nonclinical studies in mice using AMT-060, expressing WT hFIX, showed safety and dose-dependent increases in hepatic transduction, plasma hFIX protein levels, and plasma hFIX activity. NHPs dosed with 5×10^{12} genome copies (gc) per kg also showed safety and demonstrated average FIX activity levels of 9%. Treatment of macaques with the same dose of AMT-061 resulted in similar human FIX protein expression, but FIX activity was 6.5-fold enhanced, consistent with the increased activity of the Padua variant.[7]

Clinical development program

Precursor clinical studies with scAAV8-LP1-hFIXco

Nathwani et al reported the first clinical trial of scAAV-mediated gene transfer in hemophilia B in 2011.[8] Six patients with severe hemophilia B (FIX activity <1% of normal) received IV infusion of scAAV8-LP1-hFIXco at doses ranging from 2×10^{11} to 2×10^{12} vector genomes per kg. The high dose cohort was later expanded to include a total of 6 participants.[9] Overall, the trial showed that the vector was well tolerated and resulted in dose-dependent increases in FIX activity levels. All patients demonstrated stable FIX expression over the long term, with average FIX levels ~5% of normal in the high-dose cohort. These participants have maintained stable and therapeutic expression of FIX extending >10 years (as reported in 2022) with no late toxicity observed.[10]

Overview of the clinical development program

After the paramount early success by the investigators at the University College London and St. Jude Children's Research Hospital, the Dutch biotechnology company uniQure licensed the scAAV5-LP1-hFIXco technology from St. Jude. The clinical studies that supported the regulatory approval of etranacogene dezaparvovec (Table 1) began with an open label, dose-escalation trial of a precursor molecule, AMT-060.[11] The vector, manufactured in insect cells using a baculovirus expression system, was administered at 2 doses, 5×10^{12} and 2×10^{13} gc/kg. A subsequent phase 2b study of the successor investigational agent AMT-061, modified solely by the substitution of a single amino acid at position 338 (R338L), studied 3 participants at a dose of 2×10^{13} gc/kg.[12] Based on the data from the phase 2b study, the phase 3 study of AMT-061 enrolled 54 adult men in an open-label study after a lead-in period of at least 6 months on FIX prophylaxis.[6] All participants received a dose of 2×10^{13} gc/kg; the primary end point was the annualized bleeding rate (ABR) from month 7 to month 18 after infusion compared with the ABR during the lead-in period. FIX activity in the plasma and usage of FIX concentrate served as secondary end points. The completed phase 2b study and the ongoing phase 3 study provided evidence of safety and efficacy, and etranacogene dezaparvovec was approved by FDA in November 2022.

Initial studies with AMT-060

Beginning in 2015, the original sponsor, uniQure, carried out an initial study using a precursor molecule, AMT-060, to etranacogene dezaparvovec (AMT-061). These studies built on earlier trials,[1] notably the ones summarized above that used the same self-complementary LP1-hFIXco expression cassette as AMT-060[8,9] but used a different capsid, AAV5. A hypothesis of the investigators was that the AAV5 capsid would demonstrate a preferential immune profile compared with other naturally occurring serotypes. AAV5 is the least conserved compared with AAV2 and has a lower prevalence of neutralizing antibodies (NAbs) in the population compared with other naturally occurring serotypes.[13,14] Similar in design to previous studies, this study enrolled adult males with severe hemophilia B (FIX activity < 1%) and a severe bleeding phenotype, defined in the protocol as either being on prophylaxis or, for patients treated on demand, experiencing at least 4 bleeds in the previous year, or the presence of chronic hemophilic arthropathy or moderately severe hemophilia B (FIX ≥1% and ≤2%) with a

Table 1. Summary of clinical studies supporting approval of etranacogene dezaparvovec

Characteristic	Phase 1 open-label dose escalation AMT-060	Phase 2b open-label AMT-061	Phase 3 open-label AMT-061
Number of participants infused	10	3	54
Dose(s)	5×10^{12} gc/kg 2×10^{13} gc/kg	2×10^{13} gc/kg	2×10^{13} gc/kg
Ages, mean (range), y	54 (33-72)	46.7 (43-50)	41.5 (19-75)
Severity of hemophilia	9 severe (<1%) 1 moderate (1.5%)	2 severe (<1%) 1 moderate (1%)	44 severe 10 moderately severe (≤2%)
FIX prophylaxis before study entry	9 on prophylaxis 1 on demand	3 on prophylaxis	54 on prophylaxis 4 on-demand*
HIV-positive	1/10 participants	2/3 participants	3/54 participants
Previous HCV infection	6/10 participants	3/3 participants	28/54 participants
Mean FIX activity (1-stage aPTT-based FIX) after vector infusion	Cohort 1, 4.4 IU/dl (95% CI, 1.5-7.3 IU/dl) at 52 wk; Cohort 2, 6.9 IU/dl (95% CI, 2.6-11.3 IU/dl) at 26 wk	47% normal (33.2%-57%) at 26 wk	Change from baseline: 36.2% at 6 mo 38.8% at 12 mo 34.3% at 18 mo

aPTT, activated partial thromboplastin time; CI, confidence interval; HCV, hepatitis C virus.
*Some participants received both on-demand and prophylaxis during the lead-in period.

severe bleeding phenotype. The study was designed to exclude those who tested positive for preexisting NAbs to AAV5, although none were found. Based on non-clinical pharmacology studies in NHPs, the minimum anticipated biological effect level was hypothesized to be 5×10^{12} gc/kg body weight. The study tested 2 doses (5×10^{12} and 2×10^{13} gc/kg), with 5 participants enrolled at each dose. Nine of 10 participants were on prophylactic FIX infusion at baseline and were advised to continue prophylaxis for 6 to 12 weeks after vector infusion. If trough levels were ≥2% on 2 consecutive measurements after that time, participants were allowed to taper and stop prophylaxis. Investigators were instructed to prescribe a tapering course of corticosteroids if alanine transaminase (ALT) levels increased to >1.5 to 2× above baseline levels.

The findings in this Initial study largely recapitulated those previously described by Nathwani et al[8,9] in terms of FIX levels and occurrence of transaminase elevation, despite using doses that were 2.5-fold and 10-fold higher than the highest dose used in the earlier study. In the lower dose cohort, all participants saw a rise in FIX activity levels, with a mean level of 4.4 IU/dL (range 1.3-6.8 IU/dL), whereas in the higher dose cohort, FIX activity levels showed a mean level of 6.9 IU/dL (range 3.1-12.7 IU/dL). This was accompanied by cessation of prophylaxis in 4 of 5 and 5 of 5 participants in the low and high dose cohorts, respectively. For cohort 1, annualized spontaneous bleeds were reduced from a mean of 9.8 in the year before treatment to 4.6 in the year after treatment; for cohort 2, for 4 of 5 participants, annualized spontaneous bleeds were reduced from a mean of 3.0 to 0.9, with participant 10 not included because historical bleed data were not available. In terms of safety, 3 of 10 participants, including 1 in the lower dose and 2 in the higher dose, showed modest rises in ALT. With an upper limit of normal (ULN) of 40 U/L, the maximum value recorded was 85 U/L in 1 participant in the high dose cohort. As had been previously observed, the transaminase elevations were asymptomatic and the findings resolved on tapering steroids.[11] Of note, and distinct from previous findings, there was no loss of FIX activity among these 3 participants. Moreover, the capsid-specific interferon-gamma enzyme-linked immunospot assays were negative save for a single positive measurement in a participant who did not show elevated ALT. One participant with an increase in ALT showed increased levels of interferon gamma that coincided temporally with the transaminase elevation, but no reduction in FIX levels occurred. These changes were thus judged to be not clinically relevant.

Phase 2b and phase 3 studies of etranacogene dezaparvovec

The online publication of the phase 1/2 study of AMT-060[11] coincided with the first full-length report in the literature of a gene therapy study using FIX Padua.[15] This resulted in durable circulating FIX activity levels of 33.7% ± 18.5% in the 10 adult males enrolled, with no evidence of inhibitor formation despite the use of a non–wild-type FIX molecule. Although an earlier attempt to use the FIX Padua transgene had been associated with complete loss of transgene expression in 6 of 7 enrolled participants,[16] given the successful demonstration of durable expression, all sponsors, including uniQure, moved to incorporate the R338L variant; there remain no AAV-based gene replacement programs in active development using a WT FIX coding sequence.[17]

The initial testing by uniQure of AMT-061 built on the experience gained in the AMT-060 trial, and enrolled 3 participants with moderate to severe hemophilia B in a phase 2b study that assessed a single dose (2×10^{13} vector genomes/kg), identical to the higher dose in the AMT-060 study. This small study[12] provided compelling results at interim analysis at 26 weeks, with FIX activity levels of 33.2%, 51.0%, and 57.0% of normal measured using a 1-stage activated partial thromboplastin time-based assay. Of note, the 3 participants, aged 43, 50, and 47 years, all had a history of hepatitis C, resolved, and 2 of 3 were HIV positive, thus representative of the viral exposure history for their age group in the hemophilia population. Two individuals had minor increases in transaminases (1 preinfusion on the day of dosing and thus not related to vector administration), but none received a course of steroids. All 3 participants had low titer antibodies to AAV pretreatment, as determined by an anti-AAV assay that had not been validated, suggesting that these antibodies were not an impediment to effective transduction. The 3 participants were all on FIX prophylaxis at entry to the study and had nonetheless experienced 3, 1, and 5 bleeds requiring treatment in the previous year. After

vector infusion, there were no bleeds and no requirement for FIX infusion in the ensuing 26 weeks, consistent with the circulating FIX activity levels achieved.

Based on these data and the results from the phase 1 study, a phase 3 open-label, single-dose, multicenter, multinational study was conducted.[6] The overall trial design included prospective collection of data on bleeds and infusions during a 6-month run-in period on FIX prophylaxis (standard of care), followed by vector infusion and 18 months of follow-up for safety and efficacy, with subsequent follow-up planned through 5 years after vector infusion. A summary of the characteristics of this study is shown in Table 2.

Among 75 individuals screened, there were 8 screen failures (4 failed to provide consent, 3 had not been on stable prophylaxis for at least 2 months, and 1 was excluded based on hepatic exclusion criteria). Of the 67 who entered the lead-in phase, 13 were discontinued before dosing (5 based on ineligible fibroscan scores, 2 based on comorbidities, 2 based on concerns regarding the severe acute respiratory syndrome coronavirus 2 [SARS-CoV2] pandemic, 1 based on concomitant medications, and 1 based on withdrawal of consent), leaving 54 participants who were dosed. The demographics of the enrolled and dosed participants in the United States (37%) and the European Union or United Kingdom (63%) were generally reflective of the adult hemophilia B population in these geographies, although only 3 of 54 were HIV positive.

Table 2. Phase 3 study of etranacogene dezaparvovec

Current sponsor Original sponsor	CSL Behring uniQure biopharma B.V.
Vector (alternative name) and dose	AAV5-hFIXco-Padua (AMT-061) 2×10^{13} gc/kg of body weight
Approval status	Approved in United States: 22 November 2022
	Approved in European Union: 20 February 2023
Indication	Indicated for treatment of adults with hemophilia B who:
	• Currently use FIX prophylaxis therapy, or
	• Have current or historical life-threatening hemorrhage, or
	• Have repeated, serious spontaneous bleeding episodes
Trial	HOPE-B (NCT03569891)
	54 participants
	Multinational, open-label, single-arm
Inclusion criteria	Adult males
	Severe or moderately severe hemophilia B (FIX ≤ 2 IU/dL)
	Currently on FIX prophylaxis
	>150 previous exposure days of treatment with FIX protein
	With or without preexisting NAbs to AAV5
Selected exclusion criteria	FIX inhibitors before or at screening
	Select screening laboratory value >2 times upper limit of normal
	Positive HIV test at screening, not controlled with antiviral therapy
	Active infection with hepatitis B or C virus at screening
	Previous gene therapy treatment
	Receipt of an experimental agent within 60 d before screening
	Current participation or anticipated participation within 1 y after study drug administration in this trial in any other interventional clinical trial involving drugs or devices

Analysis of efficacy

Both the peer-reviewed report[6] and the FDA Summary Basis for Regulatory Action[18] provide comprehensive summaries of the safety and efficacy data. Efficacy results for the phase 3 study demonstrated a decrease in ABR from 4.19 (95% confidence interval, 3.22-5.45) during the run-in period to 1.9 (95% confidence interval, 1.0-3.4) during month 7 to month 18 after vector infusion, meeting the criteria for noninferiority compared with FIX prophylaxis. As has commonly been the case for gene therapies for single gene disorders, the evidence for efficacy with Hemgenix is compelling. The phase 3 study of 54 patients showed that 52 of 54 were able to stop prophylaxis, not surprising given a mean FIX level at 18 months after infusion of 37% (± 21%). Of the 2 that could not, 1 received only 10% of the proposed dose, because of an infusion reaction; and the other had the highest titer preexisting NAb to AAV5, a titer of 1:3212. CSL Behring will, as a post-marketing requirement, validate an assay for anti-AAV5 NAbs and carry out a study that assesses efficacy in patients with preexisting anti-AAV5 NAb. This will help to establish a cutoff for screening, which will likely further improve overall percentage of patients responding to therapy. To date, the treatment appears durable, with data available as long as 3 years after vector infusion for the phase 2b study[19] and 2 years after infusion for the phase 3 participants.[18] The only uncertainty regarding efficacy proceeds from the variability among participants, with plateau levels of expression ranging from ~5% to 112% in participants whose pretreatment NAbs to AAV5 were below the level of detection, and who all received the same vector dose (supplemental Figure 5 in Pipe et al[6]). The field is still in the process of defining the underlying mechanisms of this variability and assessing whether various vector preparations differ in the range of expression at a given dose. It is worth noting that there are a number of steps required for a viral vector to successfully mediate expression in its target cell, and each step is subject to individual variability. The vector journey includes survival in the bloodstream, in which it may encounter varying amounts of NAbs; extravasation and targeting of the hepatocyte, which may have different numbers of cell-surface receptors; cellular entry, endosomal escape, and nuclear translocation; and finally, uncoating and generation of double stranded DNA suitable for expression. Overall, though, the efficacy data are clear and contribute to the favorable benefit/risk ratio for Hemgenix.

Factors affecting FIX activity level

The FDA Summary Basis for Regulatory Action[18] analyzes factors associated with higher or lower plateau levels of FIX expression. This is useful given the variability in levels observed among patients who all received the same vector dose (see above). Some factors are expected, others less so. Patients with higher body mass index (BMI) had higher levels of FIX (32% higher for those with BMI 25-29 kg/m^2, n = 29 participants; 49% higher for those with BMI ≥30 kg/m^2, n = 10 participants) than those with normal BMI (<25 kg/m^2, n = 11 participants). This likely reflects the weight-based dosing and suggests that the number of hepatocytes (target cells) in the liver does not increase linearly with body weight. Perhaps not predicted was the finding that younger patients (aged <40 years) showed plateau levels 1.5-fold to twofold lower than those aged ≥60 years; of course, this may be related to BMI, although this is not specifically addressed in the document. Also of

note, those participants who experienced ALT elevation (see below) had 44% lower mean FIX activity at month 18 than those who did not, and those who were treated with corticosteroids for ALT elevations had 63% lower mean FIX activity. Increases in liver aminotransferase levels leading to loss of transgene expression have been previously reported.[15,20] These data would suggest that whatever phenomenon is signified by ALT elevation (and concomitant steroid administration), it is not conducive to optimal retention/expression of the transgene.

Analysis of safety

In the setting of systemically administered AAV, safety signals can be related to the vector capsid, the transgene product, or corticosteroids administered to reduce responses to the capsid. The following summary is drawn primarily from the FDA Summary Basis of Regulatory Action, and from the United States Prescribing Information.[21] The safety population consisted of the 3 participants in the phase 2b study and the 54 participants in the phase 3 study, of whom 1 received only 10% of the intended dose. The most common adverse reactions (incidence ≥5%) included ALT and aspartate transaminase elevation, headache, creatine kinase elevation, flu-like symptoms, infusion-related reactions, malaise, and fatigue. Infusion reactions occurred in 7 participants during the infusion and in 12 participants after infusion; 11 of 19 participants recovered on the same day or the day after, and the remaining 8 of 19 within 8 days. Infusions were temporarily stopped in 3 participants and restarted at a slower infusion rate after administration of corticosteroids or antihistamines, although as noted above, in 1 participant infusion was not resumed. Twenty-four participants (42%) were observed to have elevated ALT levels, mostly mild. Five participants had ALT elevations >2 to 3× ULN (89-130 IU/L), 1 participant had ALT elevation >3 to 5X ULN (193 IU/L), and 1 participant had an ALT elevation >5× ULN (275 IU/L). In 17 of 24, elevations were observed in the first 4 months after vector infusion, and 11 of 17 resolved within the first four months. Nine participants never normalized, with levels at 2-year follow up ranging from 48 to 193 IU/L. Interestingly, 7 of 24 had onset of increased ALT 6 to 24 months after vector injection; 5 of 7 had other risk factors including HIV or history of hepatitis C. Three of these 7 also failed to normalize. The basis for these persistent changes in those without a history of hepatitis or other risk factors is unknown based on published data. Relevant medical history including alcohol intake, obesity, diabetes, insulin resistance, or weight gain for individual participants might be informative to assess a potential role of alcoholic hepatitis or metabolic dysfunction–associated steatotic liver disease as the cause of these changes. Nineteen of the 24 participants with ALT elevation also had aspartate transaminase elevation. The protocol for the phase 3 study provided for administration of a tapering course of corticosteroids in the setting of transaminase elevation.[6] Nine of 24 participants with ALT elevations received a course of steroids; the mean duration of steroid administration was 80 days. There were no reported adverse events related to steroid administration. Of note, a single case of hepatocellular carcinoma (HCC) was discovered during a routine, study-mandated ultrasound in an individual with multiple risk factors for HCC. Rigorous integration site analysis revealed no evidence of AAV insertional mutagenesis as the etiology for the tumor[22] (see below).

Overall, the safety profile of AMT-061 was largely consistent with the published literature on AAV-mediated gene transfer to liver. The first trial of liver-directed, AAV-mediated gene therapy for hemophilia B was initiated in 2001.[1] Long-term follow-up of the participants, published in 2020, showed no evidence of safety concerns.[23] Similarly, the second liver-directed trial for hemophilia B,[8] begun in 2010, continues to show an absence of safety concerns.[10] Although the numbers are small, these findings would seem to bode well for this class of therapeutics. It should be noted that these earlier studies used vector doses 1 log lower than the studies of etranacogene dezaparvovec. This is relevant because animal studies suggest that risks of insertional mutagenesis, which have not been observed clinically, are dose dependent.[24] The sponsor CSL Behring will perform a voluntary, prospective, 15-year observational postmarketing study in 250 patients with Hemophilia B treated with Hemgenix as an additional pharmacovigilance measure.[18] This will add substantially to our knowledge base for long-term safety. Risks related to germline transmission of vector sequences are theoretical and have not been described in humans or other species; these risks can be mitigated by the use of barrier birth control until the vector has been cleared from the semen.

Features of special interest

Durability and variability of expression

A key metric in gene therapy for genetic disease for patients, physicians, and payers is the durability of expression. The phase 3 trial of etranacogene dezaparvovec is designed to follow participants for 5 years; these data will thus be captured. The longest duration of expression of AMT-061 published to date comes from follow-up of the phase 2b study, which shows 3 years of stable expression in the 3 participants.[19] There is reason to be optimistic on this question, based on data published earlier by Nathwani et al[10] showing durable expression for 10 years in patients treated with a similar vector (scAAV8-LP1-hFIXco). Note that although this vector expresses wild-type FIX rather than the FIX Padua variant, the amount of protein product expressed from the 2 vectors is similar, based on the difference in specific activities of the 2 proteins, auguring well for the durability of Hemgenix expression. The other key metric is variability, that is, the spread in plateau levels observed in a group of patients who all receive the same dose of vector. Given the number of factors that can affect plateau levels (see above), it is perhaps not surprising that the range is considerable. The best data addressing this point directly come from the phase 3 study,[6] in which it can be seen (supplemental Figure 5) that, among patients with no detectable preexisting NAb to AAV5, all receiving the same dose of vector, the lowest plateau FIX level was ~5%, and the highest was ~112%. Although levels within this range are all likely to improve the hemophilic phenotype compared with the patient's baseline, the range is clearly quite large. A narrower range of plateau FIX activity levels is a desirable goal in this indication.

The role of preexisting NAbs to the AAV5 capsid

One of the most notable differences of this study compared with most of the trials in which AAV is administered systemically is the inclusion of participants with preexisting NAbs. Early clinical investigation showed that anti-AAV NAbs, even at modest titers, prevented successful liver transduction when the vector was delivered through the circulation.[1] This observation has been consistently replicated in animal models[25] as well as clinical studies and served as the rationale for excluding individuals with

preexisting NAbs from participation, including in other clinical trials using AAV5,[26] and indeed in the initial study of AMT-060.[11]

However, for reasons that are not completely understood, the AAV5 capsid appears to be less sensitive to neutralization by preexisting NAbs. A previous clinical study had shown no correlation between the presence of preexisting anti-AAV5 NAbs and the therapeutic efficacy of AMT-060 in 10 patients with hemophilia B, because participants with anti-AAV5 NAb titers of up to 350 achieved therapeutic hFIX activity levels after AMT-060 administration.[27] Similarly, preexisting anti-AAV5 NAb titers up to 1030 had no effect on liver transduction or hFIX protein expression in NHPs treated with AMT-060.[27]

In the HOPE-B study, preexisting neutralizing anti-AAV5 antibodies were assessed using an unvalidated clinical trial assay but were not used as an exclusion criterion. Of the 54 participants, 21 of 54 participants (38.9%) had anti-AAV5 NAbs before AAV administration. Twelve months after AAV, the average FIX activity was 42% ± 22% for individuals with NAb titers of ≤1:100 (n = 45) and 36% ± 17% for those with NAb titers ranging from >1:100 to <1:700 (n = 5). In participants with NAb titers ≤1:350 (n = 47), the mean FIX activity at month 12 was 42% ± 22%, whereas it dropped to 27% ± 17% in participants with NAb titers >1:350 to <1:700 (n = 3). Although the data set is limited, and the anti-AAV NAb titer assay used in this study has not been validated, these results seem to suggest AAV5 is less sensitive to neutralization by preexisting NAbs but not completely resistant. It is worth noting that no human FIX expression was observed in the only participant who had a titer >700, and exogenous FIX prophylaxis had to be restarted because hemostatic protection was not achieved. Importantly, adverse events were similar among participants with or without AAV5 NAbs, with no consistent association between anti-AAV5 NAbs and safety.

The US FDA has mandated 2 pivotal postmarketing requirement studies to assess the risk of bleeding due to decreased therapeutic efficacy in the presence of preexisting anti-AAV5 NAbs. The first is a study to validate a sensitive assay for detecting anti-AAV5 NAbs. The second required study will enroll 35 patients with hemophilia B, at least 10 of whom have preexisting NAb titers ≥1:1400, to receive Hemgenix to examine the association between bleeding risk and preexisting anti-AAV5 NAbs, once a validated assay is available.

The reasons behind an apparent serotype-specific difference in the effect of NAbs on systemic AAV gene therapy warrants further investigation. Some authors have suggested that significant variations in the avidity of preexisting anti-AAV immunoglobulin G's (IgGs) among serotypes may, at least in part, explain the observations above. Specifically, Majowicz et al described that anti-AAV2 and anti-AAV8 IgGs formed stronger complexes with their respective antigens, whereas anti-AAV5 IgGs displayed weaker binding.[28]

Preexisting NAbs pose 2 key challenges to systemically administered AAV gene therapy: accurate identification of those with NAbs, including an appropriate cutoff; and method(s) to eradicate antibodies if present. Investigators have assessed diverse mitigation strategies, including plasmapheresis[29] and immunoadsorption,[30] to physically eliminate antibodies from the circulation. The use of an IgG-cleaving endopeptidase, currently approved as a treatment for prevention of renal transplant rejection, offers another approach to enable eradication of preexisting antibodies.[31] However, none of these techniques have been approved in the clinic yet to enable the inclusion of participants who would otherwise be excluded from enrollment due to preexisting immunity.

Clearing of vector from the semen

The first clinical trial of liver-directed AAV gene therapy revealed an unexpected (based on nonclinical studies) finding of vector in the semen on routine studies of body fluids after vector administration.[1] The theoretical risk of vector in the semen is that offspring could be transgenic for the donated gene, and the exogenous DNA could disrupt the complex program of gene activation and repression that characterizes normal fetal development. Subsequent development of a rabbit model recapitulated the finding of vector in the semen and showed that clearance of vector was time and dose dependent.[32,33] This risk is now routinely mitigated by the use of barrier birth control, ideally until 3 consecutive semen samples show measurements below the level of detection for vector DNA in the semen. For Hemgenix, data showed that by 24 months after administration, 87% of participants had reached absence of vector DNA in the semen, as judged by two consecutive semen samples below the level of detection for vector DNA.[18] This relatively long time to clear likely reflects the higher dose used here; as a comparison, the time to clear semen, judged by 2 serial negative samples, for another liver-directed AAV trial administered at a 40-fold lower dose, was 16 weeks for 100% of participants.[15]

Risk of insertional mutagenesis

Recombinant AAV (rAAV) vectors, unlike wild-type AAV, lack the machinery needed to mediate active integration into the host cell genome and primarily remain as circular episomes in the cell nucleus.[34] However, abundant data from animal studies, and from a limited number of clinical studies, have shown that AAV vectors can integrate at low frequency into the target cell genome,[35-37] with some studies suggesting that rAAV integration is dependent on preexisting double strand breaks in the genome.[38] The consensus view on AAV integration, as summarized by The American Society of Gene and Cell Therapy Working Group on AAV Integration is that "the frequency of AAV integration is low and the risk of malignancy appears to be theoretical given that no cases of cancer associated with rAAV have been reported in humans to date."[39] The working group noted that given the current lack of observed genotoxicity in large animal models and humans, routine liver biopsies without medical indication should be avoided, and recommended patient follow-up should include hepatic ultrasound scans or magnetic resonance imaging and alpha-fetoprotein levels annually for the first 5 years after vector administration.

The number of studies assessing AAV integration in clinical trials is limited. Analyses of human biopsies after AAV gene transfer have confirmed that AAV integration is low in frequency.[37,40] Recently, a participant who had received etranacogene dezaparvovec was diagnosed with HCC 1 year after AAV administration, on a study-required routine liver ultrasound. Resection of the solitary lesion and adjacent normal tissue showed 3.2 vector copies per cell in the tumor and 4.1 vector copies in the cell in the normal adjacent tissue. Integration site analysis showed 56 unique integration sites in the HCC and 39 in the HCC-adjacent normal tissue; overall, vector integration events were found in <0.03% of cells. In both samples, no dominant integration sites were found. If HCC development were driven by AAV vector integration, frequent integration events,

with at least 1 dominant event, would be expected. Neither was observed in the patient's samples. Of note, the patient had a prior history of hepatitis virus infection and advanced age, both of which are risk factors for HCC. In conclusion, the molecular and vector integration analysis of the index case of HCC after liver-directed rAAV-based gene therapy determined that AAV was unlikely to have contributed to HCC.[22]

The current lack of observed rAAV-associated tumorigenesis in large animals and humans suggests that the risk is low compared with neonatal mice. If the mice data do indeed extrapolate to humans, then the most provocative clinical test for the potential risk of insertional mutagenesis in humans is illustrated by Zolgensma (onasemnogene abeparvovec-xioi), an approved AAV therapy for the treatment of spinal muscular atrophy. Zolgensma is given at a very high dose (the recommended dose is 1.1×10^{14} vector genomes/kg of body weight administered as an IV infusion) to pediatric patients aged <2 years. In addition, it contains a very strong, ubiquitous promoter. Importantly, to date, >1800 patients have been treated with Zolgensma, with no reports of HCC.

Overall, although there is consensus in the field that additional studies are warranted to better understand the risks associated with AAV integration, and that long-term follow-up studies are needed to continue to fully assess the safety profile of AAV products, the results discussed above suggest that the theoretical risk of genotoxicity associated with rAAV chromosomal integration after administration of AAV in humans is very low and does not outweigh the potential benefit from this therapy, especially for serious and life-threatening conditions.

Future perspectives

The recent approval of etranacogene dezaparvovec undoubtedly represents a landmark achievement for the gene therapy field. However, there remain significant challenges to extend gene therapy for hemophilia to a broader population, including pediatrics. For instance, the durability of transgene expression is a critical aspect to consider. Although no indication of waning transgene expression in adult patients who have been treated with AAV has been recorded in hemophilia B studies, a decrease in FIX levels in younger pediatric patients due to liver growth would be expected with a (predominantly) nonintegrating vector. The development of effective gene therapies for pediatrics is likely to include integrative and/or redosable vectors. Similarly, decreasing the variability in FIX levels, for example, through a gene editing approach at the endogenous genomic location or via readministration to achieve optimal levels, is a key factor that needs to be addressed in future research.

Authorship

Contribution: K.A.H. and X.M.A. drafted parts of the manuscript separately, edited the manuscript, and approved the final version.

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Correspondence: Katherine A. High, Rockefeller University Therapeutics, 1230 York Ave, New York, NY 10065; email: khigh@rockefeller.edu.

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