Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B

Short title: Gene therapy with AAV5-hFIX in hemophilia B

Wolfgang Miesbach[1], Karina Meijer[2], Michiel Coppens[3], Peter Kampmann[4], Robert Klamroth[5], Roger Schutgens[6], Marco Tangelder[7], Giancarlo Castaman[8], Joachim Schwäble[9], Halvard Bonig[9,10], Erhard Seifried[9], Federica Cattaneo[11], Christian Meyer[12], Frank W.G. Leebeek[13]

[1]University Hospital Frankfurt, Frankfurt, Germany; [2]University Medical Centre Groningen, University of Groningen, Groningen, the Netherlands; [3]Academic Medical Center, Amsterdam, the Netherlands; [4]Rigshospitalet, Copenhagen, Denmark; [5]Vivantes Klinikum, Berlin, Germany; [6]University Medical Centre Utrecht and University Utrecht, Utrecht, the Netherlands; [7]Formerly uniQure biopharma, B.V., Amsterdam, the Netherlands, currently Medpace, Cincinnati, Ohio, United States of America; [8]Azienda Ospedaliera Universitaria Careggi, Florence, Italy; [9]Institute for Transfusion Medicine and Immunohematology, Goethe University Hospital Medical School, German Red Cross Blood Donor Service, Frankfurt am Main, Germany; [10]Department of Medicine/Hematology, University of Washington, Seattle, WA; [11]Chiesi Farmaceutici S.p.A., Parma, Italy; [12]Formerly uniQure biopharma, B.V., Amsterdam, the Netherlands, currently Therachon AG, Basel Switzerland; [13]Erasmus University Medical Centre, Rotterdam, the Netherlands.

Corresponding author: Professor F.W.G. Leebeek M.D. Ph.D., Erasmus University Medical Centre, Rotterdam, the Netherlands. Email: f.leebeek@erasmusmc.nl

Key points

- AAV5 liver-directed wildtype hFIX gene transfer was well tolerated and clinically effective in severe and moderate-severe hemophilia B.
- No cellular immune responses to the vector were detected and FIX expression levels were stable over the entire observation period.

Abstract

Hemophilia B gene therapy aims to ameliorate bleeding risk and provide endogenous factor IX (FIX) activity/synthesis through a single treatment, eliminating the requirement for FIX concentrate. AMT-060 combines an adeno-associated virus-5 (AAV5) vector with a liver-specific promoter driving expression of a codon-optimized wild-type human FIX gene. This multi-national, open-label study included ten adults with hemophilia B (FIX ≤2% of normal) and severe-bleeding phenotype. No participants tested positive for AAV5-neutralizing antibodies using a green-fluorescent protein-based assay and all 10 were enrolled. A single dose of $5x10^{12}$ or $2x10^{13}$ genome copies of AMT-060/kilogram was administered to five-participants each. In the low-dose cohort, mean endogenous FIX activity increased to 4·4 IU/dL. Annualized FIX use was reduced by 81%, and mean annualized spontaneous bleeding rate (ASBR) decreased from 9·8 to 4·6 (53%). In the higher-dose cohort, mean FIX activity increased to 6·9 IU/dL. Annualized FIX use decreased by 73%, and mean ASBR declined from 3·0 to 0·9 (70%). There was no reduction in traumatic bleeds. FIX activity was stable in both cohorts and eight of nine participants receiving FIX at study entry stopped prophylaxis. Limited, asymptomatic, and transient alanine aminotransferase elevations in the low-dose (n=1) and higher-dose (n=2) cohorts, were treated with prednisolone. No decrease in FIX activity or capsid-specific T-cell responses were detected during transaminase elevations. A single infusion of AMT-060 had a positive safety profile and resulted in stable and clinically-important FIX activity increases, a marked reduction in spontaneous bleeds and FIX concentrate use, without detectable cellular-immune responses against capsids.

ClinicalTrials.gov NCT02396342

Introduction

Hemophilia B is a monogenic X-linked recessive coagulation disorder causing a deficiency of coagulation factor IX (FIX) and affecting approximately 25,000 individuals globally.[1-3] The natural course of individuals with the severe phenotype of hemophilia B (FIX <1 U/dL) is characterized by lifelong spontaneous hemorrhages into joints, soft tissues, and muscles;[2] typically leading to disabling synovitis, crippling arthropathy, and muscle atrophy.[4] Treatment consists of intravenous factor replacement by injection of purified plasma-derived or recombinant FIX,[2] which is inherently expensive, requires complex infrastructure, and is not available in some countries.

Factor IX concentrate can be administered on-demand or prophylactically. On-demand therapy at the time of a bleed is effective at stopping individual hemorrhages, but does not prevent occurrence of bleeds. Thus, by comparison, prophylaxis, which consists of regular infusions of FIX concentrate given up to three times weekly to prevent (spontaneous) bleeding, is associated with better joint health outcomes[2,5]. However, low trough plasma levels may not optimally protect against bleeds, sometimes subclinical, in the hours just prior to subsequent factor infusion.[6] Prophylaxis is expensive, with one study estimating individual annual costs of over €135,000,[7] and burdensome, requiring lifelong multiple weekly intravenous infusions from early childhood.[8]

Gene transfer potentially offers constant and sustained endogenous production of functional FIX. Gene transfer is particularly attractive for the treatment of hemophilia since even a modest rise in clotting factor activity can substantially attenuate the bleeding risk.[9] Durable FIX expression was recently demonstrated in ten participants who received a single infusion of adeno-associated virus (AAV) serotype eight vectors containing a wildtype FIX gene with a liver-specific promoter (LP1).[10,11] A dose-dependent increase in circulating FIX was established, with a mean FIX activity of 5·1% lasting for at least four years. In the highest dose cohort, the annual use of FIX replacement therapy and bleeding rate were both reduced by 90%.[11] However, treatment efficacy was limited by apparent activation of T-cells in response to the viral capsid in four of six participants in the high-dose group

(2×10^{12} genome copies [gc] per kg), resulting in clearance of transduced hepatocytes and consequential loss of 50-70% of FIX activity. This activation accompanied an otherwise asymptomatic, transient elevation of alanine aminotransferase (ALT), which normalized after a short course of prednisolone.[11] A similar loss of transgene activity has been reported following gene transfer with AAV2.[12]

These data provided the rationale for development of the novel gene therapy vector tested in this trial. AMT-060 combines the previously tested human wildtype FIX gene cassette with an AAV5 capsid.[10,11] AAV5 is capable of transducing liver tissue[13] and may offer a preferential immune profile. The prevalence of neutralizing antibodies (NAb) was lower for AAV5 versus AAV2 (3.2% versus 59% in one study and 18% versus 30% in another)[14,15] and for AAV5 versus AAV8 (3.2% and 19%, respectively),[14] although it should be noted that prevalence rates vary by population and region. Additionally, AAV5 does not appear to elicit cellular immune responses against the capsid,[16] which may distinguish it from other AAV serotypes. Here, we report the planned interim analysis of a 5-year Phase 1/2 study. These data reflect the safety and efficacy of AMT-060 in adults with hemophilia B with FIX activity <2% with a follow up of up to 1-year.

Materials and methods

Study design and participants

This multi-national, open-label, Phase 1/2, dose-escalation study included ten adults with hemophilia B (FIX activity ≤2% of normal) receiving either prophylactic FIX, or on-demand FIX with ≥4 bleeds/year or hemophilic arthropathy (ClinicalTrials.gov NCT02396342; EudraCT number 2013-005579-42). Participants were male, aged ≥18 years, with either a) severe FIX deficiency (FIX < 1%) and a severe bleeding phenotype or b) moderately severe FIX deficiency (FIX ≥ 1% and ≤ 2%) and a severe bleeding phenotype. Individuals positive for pre-existing NAb to AAV5 were excluded. A sample was considered positive at 29% inhibition of transduction compared with the negative control (pooled NAb-negative human sera). Full inclusion/exclusion criteria are listed in Supplementary Table 1. The study was approved by the Institutional Review Board/Institutional Ethics Committee at each center. All participants provided written informed consent. The trial was performed according to the Declaration of Helsinki and the principles of Good Clinical Practice.

AMT-060

AMT-060 is a novel gene transfer product that consists of an AAV5 vector incorporating a small gene cassette containing codon-optimized wildtype hFIX under the control of a liver-specific promoter LP1,[10,11] which permits formation of self-complementary vectors. The vector was manufactured in insect cells using a baculovirus expression system in accordance with Good Manufacturing Practices.[17] Vector titer was measured using a conventional quantitative polymerase chain reaction (QPCR)-based approach with plasmid DNA as the primary reference. The method is similar as those described for the characterization of internationally accepted AAV reference standards.[18,19]

Procedures

AMT-060 was administered as a single 250 mL peripheral intravenous infusion over 30 minutes. As this was a first-in-human study, participants were monitored at the clinical trial site for 24-hours following administration. Participants were treated in two consecutive, escalating dose cohorts: Cohort 1 (n=5) $5x10^{12}$ gc/kg (low dose) and Cohort 2 (n=5) $2x10^{13}$ gc/kg (high dose) (Figure 1). Following administration of AMT-060 to the first two participants in each cohort, the Data Monitoring Committee (DMC) evaluated available safety data before the next participant was treated. The decision to progress to Cohort 2 was made after the completion of Cohort 1 dosing and a DMC safety review. Participants remained on their pre-study regimen of FIX prophylaxis following AMT-060 administration until 6-12 weeks' post-treatment. At that time, participants who achieved FIX activity ≥2·0 IU/dL (trough levels) for at least two consecutive measurements tapered their FIX replacement therapy during a 2-week period. Continued withholding of prophylactic FIX replacement therapy was encouraged if FIX activity ≥2·0 IU/dL was maintained after tapering for at least two consecutive measurements. Guidance was provided to investigators about when to re-measure FIX based on FIX activity (1-2 days for 2·0-2·9 IU/dL up to 4-5 days for ≥8·0 IU/dL). A tapering course of corticosteroids was recommended if ALT levels increased to greater than 1·5- to 2-fold of baseline levels, in the absence of alternative etiology.

Outcome measures

Safety outcomes included treatment-related adverse events (TRAE), NAb to AAV5, total IgM and IgG antibodies against AAV5, AAV5 capsid-specific T cells, antibodies to FIX (including inhibitors), shedding of AMT-060 vector, and inflammatory markers (interleukin [IL]-1β, IL-2, IL-6, interferon [IFN]γ, monocyte chemotactic protein [MCP-1]) (Supplementary Table 2). Serious adverse events (SAEs) were defined as events that resulted in death, were life-threatening, required hospitalization or prolongation of existing hospitalization, resulted in persistent or significant disability or incapacity, congenital anomaly or birth defect, or were judged medically important by the

investigator. Adverse events were categorized as mild (awareness of symptoms, sign, illness or event that is easily tolerated), moderate (discomfort sufficient to cause interference with usual activity) or severe (incapacitating with inability to work or undertake further normal activities).

Efficacy outcome measures included FIX concentrate use as well as endogenous FIX plasma activity and protein levels measured ≥10 days after FIX concentrate use (Supplementary Table 2). Details of biochemical assessments are given in the Supplementary Methods. Bleed details including circumstances, location, severity, and treatment were assessed. Retrospective data on bleeds and FIX use were collected from participant diaries and hospital records for the year prior to study entry. Bleed data and FIX consumption after gene transfer were recorded prospectively by the participants using an electronic diary, and reviewed by the physician at study visits. Study visits occurred weekly (Cohort 1), or twice weekly (Cohort 2), up to week 12; every 2 weeks from week 12 to week 26 and quarterly between six-months and one-year. The study will continue with quarterly visits until year 3, and twice-annual visits from year 3–5 post treatment. Joint status was assessed using the Hemophilia Joint Health Score version 2·1.[20]

Immunological analyses

Details of immunological analyses are provided in the Supplementary Methods.

Data analysis

This report represents a planned interim analysis with data current to 08 November 2016; further analyses of this same population are planned to provide a total of 5-years follow up. Categorical data are presented with number of cases and percentage of total number of cases. Continuous data are shown as a mean with corresponding 95% confidence interval (CI) or standard deviation (SD) or as a median with minimum and maximum as appropriate. Due to the sample size, no formal statistical analysis was performed. Other details of the data analyses are given in the Supplementary Methods.

Results

Demographic and baseline characteristics

Ten participants from the Netherlands (n=6), Germany (n=3) and Denmark (n=1) were enrolled. There were no screening failures due to pre-existing AAV5 NAb. Nine participants were classified as having severe (<1·0 IU/dL FIX activity) hemophilia B and one participant in Cohort 1 had moderate hemophilia B (FIX activity of 1·5 IU/dL). Nine participants were on FIX prophylaxis prior to AMT-060 treatment and one participant with severe hemophilia (Cohort 2) was using on-demand therapy as needed. Participants enrolled in Cohort 1 were generally older, had more severe arthropathy, and, despite prophylactic FIX (average 4000 IU weekly), experienced more bleeds in the year prior to study entry compared with those in Cohort 2 (Table 1). The genotype of each participant is listed in Supplementary Table 3.

Safety

Adverse events

Three participants in each cohort (participants 1, 3 and 5 [Cohort 1] and participants 6, 7 and 10 [Cohort 2]) experienced a total of 14 treatment-related adverse events (TRAEs; four in Cohort 1 and ten in Cohort 2). Most related events were classified as mild, some as moderate (Table 2). Seven of the TRAE were experienced by participant 6 (liver enzyme increase [n=2], pyrexia, anxiety, palpitations, headache, and rash). The liver enzyme increase may reflect a single event as the original ALT elevation of 55 U/ml (normal range 10-50 U/mL) was recorded as recovered on day seven (51 U/mL), however, the following day ALT was 66 U/mL and prednisolone treatment was initiated. There were three protocol-defined serious adverse events (SAE) classified as possibly- or probably-related to treatment: mild, asymptomatic elevations in liver enzymes (participant 1); short, self-limiting fever in first 24 hours post-AMT-060 (participant 3), and ALT elevation (participant 6).

Liver biomarker abnormalities (elevated transaminases)

Three participants experienced mild asymptomatic elevations in ALT not associated with changes in FIX activity (Supplementary Figure 1). Participant 1 (Cohort 1) had an ALT elevation with a peak of 61 IU/L at week 10 (upper limit of normal 40 IU/L). Participant 7 (Cohort 2) experienced an ALT elevation, with peaks of 54 IU/L (week 6) and 81 IU/L (week 9). The first ALT peak coincided with concomitant ciprofloxacin treatment, while the second peak coincided with alcohol consumption. In participants 1 and 7, ALT values returned to normal within 2 and 6 weeks, respectively, of starting a course of tapering prednisolone. Participant 6 (Cohort 2) had a mild asymptomatic elevation of ALT between week 4 and week 22 that peaked at 85 IU/L at week 16 and was treated with a tapering regimen of prednisolone. The participant was weaned from prednisolone between week 18 and 26. ALT values reached the normal reference range by week 26. FIX activity remained stable throughout the duration of ALT elevations in all three patients.

Immune and inflammatory biomarkers

No participants had pre-existing NAb to AAV5 based on a functional inhibition assay. In a separate assay to determine Ig protein levels, participants 3 and 4 had low titers of pre-existing IgG antibodies against AAV5. Participant 1, participant 7, participant 8, participant 9 and participant 10 had low-titers of pre-existing anti-AAV5 IgM antibodies. As expected, all participants developed a humoral immune response to AAV5 within 1-week of gene transfer (Supplementary Figure 2). No participants developed inhibitors to FIX. There were no detectable signs of sustained AAV5 capsid-specific T-cell activation. A single T-cell measurement of 33 spot-forming units (SFU) per one million peripheral-blood mononuclear cells (PBMCs) in response to AAV5-capsid peptide pools, which was marginally above the threshold for positivity of 17, was detected in participant 3 at week 9 (Supplementary Figure 3). The increased ALT (weeks 7-26) in participant 6 coincided with the increase of an inflammatory marker (IFN-γ, weeks 3-17; Supplementary Figure 1 and Supplementary Figure 4), however, in the absence of cytotoxic immunity against transduced hepatocytes, or overt clinical

signs and symptoms, this did not appear to be clinically relevant. No other clinically-relevant changes in inflammatory biomarkers were observed in either cohort.

Detection of vector DNA

In Cohort 1, shedding of vector DNA was detected in nasal secretions (to week 18), saliva (to week 20), feces (to week 16), and urine (to week 11), semen (to week 48) and whole blood (through last assessment) (Supplementary Figure 5). In Cohort 2, shedding of vector DNA was detected in nasal secretions (to Week 12), saliva (to Week 16), feces (to Week 20), and urine (Week 22), semen (to Week 22) and whole blood (through last assessment) (Supplementary Figure 5).

Endogenous FIX activity after gene transfer

In Cohort 1, residual endogenous FIX activity increased from <1·0 IU/dL (n=4) or 1·5 IU/dL (n=1) to a mean of 4·4 IU/dL (95% CI 1·5-7·3, n=5) at 52 weeks, with four out of five participants achieving mean FIX levels ≥2·0 IU/dL (range 3·0-6·8 IU/dL) (Table 3). In Cohort 2, residual endogenous FIX activity increased from <1·0 IU/dL to a mean of 6·9 IU/dL (95% CI 2·6-11·3, n=5) at 26 weeks, with four of five participants achieving mean FIX levels >5·0 IU/dL (Table 3). FIX activity levels remained stable for the duration of follow up in both cohorts (Figure 2). Consistent with activity, mean FIX protein concentrations varied between 1·3 and 12·3% in eight of the ten participants; two participants in Cohort 1 and Cohort 2 had mean FIX protein concentrations above 50% during the 1-year and 26-week follow up periods, presumably due to genetic mutations that result in full production of a non-functional protein (Supplementary Table 4). Disease severity improved in all participants: severe to mild (n=5), severe to moderate (n=4), and from moderate to mild (n=1) (Table 3).

Exogenous FIX concentrate use and bleeding

After treatment with AMT-060, eight of the nine participants who had been on FIX prophylaxis at the time of study entry stopped FIX prophylaxis. Participant 3 in Cohort 1 remained on FIX prophylaxis per protocol with endogenous (trough) FIX activity levels <2 IU/dL (Table 3). The total annualized reduction of exogenous FIX use post-AMT-060 treatment was 79% overall (81% in Cohort 1 and 73% in Cohort 2) (Figure 3).

Annualized spontaneous bleeds were reduced from a mean of 9·8 in the year prior to AMT-060 to 4·6 in the year after treatment for participants in Cohort 1, corresponding to a 53% reduction, while total bleeds were reduced by 48% (Figure 3). In 4 participants in Cohort 2, annualized spontaneous bleeds were reduced from a mean of 3·0 to 0·9, a 70% reduction, and total bleeds were reduced by 49% (Figure 3). Mean annualized traumatic bleeds remained stable in both cohorts (2.8 vs 2.9 and 1.0 vs 1.1 pre- and post-AMT-060 in cohorts 1 and 2, respectively). Participant 10 was not included in the calculation as historical bleed data was not available. This participant, however, did not experience any bleeds post-intervention. All post-intervention bleeds were classified as mild/moderate severity by the treating physician.

Discussion

In this successful study of liver-directed gene therapy for hemophilia, ten patients with moderate (n=1) or severe (n=9) hemophilia B underwent gene transfer with AMT-060: nine participants on FIX prophylaxis and one participant receiving on-demand FIX. Gene transfer was well tolerated with no severe TRAEs. Following a single administration, FIX activity increased to levels classified as mild in six and moderate hemophilia in four participants and remained stable for the duration of follow up. Routine prophylaxis was discontinued in eight out of nine participants who required prophylaxis prior to gene therapy, resulting in large reductions in annualized factor consumption from approximately 2·64 million to 563,507 IU, a total saving of approximately 2·1 million units across all ten participants. These data are underscored by self-reported data indicating that participants

experienced fewer spontaneous bleeds following AMT-060 than they had previously while on FIX

prophylaxis. Cohort 1 was an older population (median 69 years) with severe arthropathy prior to

gene transfer (median hemophilia joint health score 27). In this group, it is likely that the ABR was

still relatively high post-treatment (4.6), due at least in part, to pre-existing joint damage. It will be of

interest in this population whether continuing improvements in arthropathy and decrease of ABR

will be observed with longer-term follow up. In contrast, Cohort 2 was much younger (median 35

years) population with lower pre-existing joint damage (median hemophilia joint health score 6). In

this population, spontaneous bleeds were much lower pre-treatment and ABR was reduced to 0.9

post-treatment. Mild elevations in liver transaminases occurred in three participants and were

treated with prednisolone, but resolved without loss of FIX activity or other detectable indication of

ongoing immune response, toxicity, or other response against AMT-060 or transduced hepatocytes.

Importantly, no participants failed screening due to pre-existing NAb against AAV5, although two

participants (3 and 4) and five participants (1, 7, 8, 9, 10) had low titers of anti-AAV5 IgG and anti-

AAV5 IgM at baseline. AMT-060 was well-tolerated and effective in all participants including those

with low-titer antibodies, suggesting no likely impact of low-titer IgG or IgM on transduction, clinical

effectiveness or cellular immune response following treatment. This initial observation will need to

be confirmed in further studies. Following treatment, two or more consecutive observations of

elevated inflammatory cytokines were reported in participant 1 (IL-2 and IFN-γ) and 6 (IFN-γ). In

both cases these elevations were transient and did not affect FIX activity. Vector DNA shedding was

detected in whole blood until the last assessment and to week 48 in semen. This is longer than

reported in the trial of scAAV2/8-LP1-hFIXco, in which clearance was reported by day 60.[12] Likewise,

in a trial utilizing a similar AAV5 vector at similar doses used in this study, shedding became

undetectable by day 30.[16] The AAV vector is non-pathogenic, cannot replicate and shed DNA is non-

infectious, so the risk for third parties such as family and healthcare personnel from prolonged

shedding is considered to be marginal. For most bodily fluids a trend towards dose-dependency was

observed, with higher mean levels of shedding in the higher-dose group. This trend was least clear in

semen, in which mean shedding was numerically lower in the higher-dose group compared with the lower-dose group at 8/12 timepoints, possibly related to the difference in age between the two cohorts. This is consistent with an earlier study by Manno et al., which reported that younger trial participants cleared vector DNA from semen more rapidly than older participants.[12]

The absence of FIX-activity loss and detectable AAV5 capsid-specific T-cell responses associated with the ALT elevations contrast with previous reports of gene transfer utilizing AAV8 and other novel capsid constructs.[10,21,22] Based on these past reports the prevailing hypothesis is that elevated ALT reflects cellular immune responses against transduced hepatocytes, resulting in cell destruction and loss of transgene expression.[23] Although we cannot exclude the possibility that the elevations observed in the present study may reflect immune responses to the vector capsid that we were unable to detect despite using the current 'gold standard' assay for T-cell activation, it is notable that no decreases in FIX activity occurred during transaminase elevations, raising the possibility of a different etiology for the this phenomenon with AAV5. This lack of detectable capsid-specific T-cell responses is compatible with a previous study of AAV5 vector gene transfer of porphobilinogen deaminase for acute intermittent porphyria, which reported no anti-AAV5 cellular immune responses.[16] Similarly, increases in ALT without T-cell activation or loss of transgene expression were preliminarily reported in a study utilizing an AAV5-FVIII construct in participants with hemophilia A.[24] The sole similarity between the three participants with ALT elevations in our study was a stronger increase in FIX protein after gene transfer. This may indicate a possible mechanistic cause such as leakage resulting from endoplasmic reticulum (ER) or other cellular stress,[25-27] particularly as rAAV vectors have been reported to activate unfolded protein response signaling pathways, suggesting ER stress, during the course of intracellular trafficking.[25] It is likely that only a fraction of all hepatocytes are transduced by the vector, so it is possible that the subset of transduced hepatocytes could experience "cellular stress" even when circulating levels of FIX do not appear to be excessively high. Continued evaluation of data emerging from multiple ongoing clinical studies of liver-directed gene transfer will help illuminate this issue.

AMT-060 utilizes the same wildtype FIX gene cassette and LP1 promoter previously described by Nathwani and colleagues in a trial of AAV8-mediated gene transfer.[10,11] Similar levels of steady state FIX activity were achieved in this study with an AAV5 vector (mean 4.4 IU/dL in Cohort 1 and 6.9 IU/dL in Cohort 2) compared with the previous study with an AAV8 vector (mean 5.1%), although the highest dose of AAV5 vector used ($2x10^{13}$ gc/kg) was greater than the highest dose of the AAV8 vector used ($2x10^{12}$ gc/kg).[11] Studies have shown that a gain-of-function FIX transgene (FIX Padua) increased the efficacy of liver-directed gene therapy with AAV at least 6 to 10-fold in murine[28] and canine[29] hemophilia models. Preliminary reports of two AAV vectors containing FIX Padua in early clinical trials indicate increases in group mean FIX values of from <2% to approximately 29% in one, and from <2% to up to 45% in another.[30,31] Interestingly, as the FIX Padua variant is known to increase specific activity by 5-10-fold, this suggests that these vectors achieved similar transgene expression levels as observed in the present study.[32,33] In the one study with available long-term results, sustained transgene expression at 1 year was only observed in 2 of 8 participants and only 1 participant had sustained expression at 2.5 years.[31] As of 1-year (Cohort 1) and 6-months (Cohort 2), FIX activity levels are stable in our participants. Nathwani and colleagues recently reported sustained FIX expression from this gene cassette at five years and beyond.[34] Monitoring will be continued for 5 years in our study as well to follow the long-term efficacy and safety.

We observed a modest dose response, with participants in our Cohort 2, who received a higher vector dose ($2x10^{13}$ gc/kg) more consistently achieving FIX activity above 5 IU/dL (four participants in Cohort 2 vs two in Cohort 1). Despite the 4-fold increase in dose, mean FIX activity increased by approximately 1.6-fold in the higher-dose group. The modest dose response observed in this study corresponds with the Nathwani et al. study, in which large increases of dose from $2x10^{11}$ to $6x10^{11}$ and $2x10^{12}$ gc/kg did not result in correspondingly large increases in FIX activity (1.8%, 2.5% and 5.1%, respectively).[11] The gene therapy vector and associated transgenic protein are dependent on complex interactions with multiple endogenous pathways and functions to realize the therapeutic effect. Furthermore, understanding of how individual variations in native physiology may impact

treatment outcome is lacking. It is possible that there may be factors limiting transgene production that dose increases do not fully overcome, for example, biodistribution of the vector away from the vessels in order to transduce a higher proportion of hepatocytes.

Participants in Cohort 1 were notably older with poorer bleed control despite prophylaxis, more extensive joint damage at study entry and were more likely to have medically-significant comorbidities than those in Cohort 2. This makes direct comparison of clinical outcomes between the two dose cohorts challenging but offers the valuable opportunity to observe the impact of gene transfer in this heterogeneous population. The reduction in spontaneous bleeds was lower (53%) in Cohort 1 compared with Cohort 2 (70%). It will be of interest whether this difference is maintained as the follow-up period extends. Interestingly, over the course of the year following gene transfer in Cohort 1, there was a decrease in bleeds culminating in cessation of spontaneous bleeds. This pattern has not been observed in Cohort 2 at six months follow up; rather, spontaneous bleeds decreased immediately with only a single reported spontaneous bleed after the end of prophylaxis. Bleed data were self-reported by the participants and it is possible that bleeds in Cohort 1 may have been over-reported and self-treated due to participants' caution, which waned as participants adjusted to a life without FIX prophylaxis. We also noticed increased physical activity shortly after AMT-060 treatment, causing traumatic bleeds in some patients and possibly underlying the lack of treatment impact on annualized traumatic bleed rates at up to 1-year follow-up. In addition, given the degree of joint disease at study entry in Cohort 1, it is possible that the decrease in bleeds over time represents gradual resolution of joint damage or decreased vessel fragility with long-term hemorrhagic control. However, while these data indicate reduced bleeding over time, there are many potential confounding factors including potential lifestyle and activity changes post-gene transfer. Therefore, these findings require further confirmation during long-term follow-up.

The strengths of this study include strict adherence to the protocol, as AMT-060 is administered as a single infusion and all participants remain in the study; rigorous follow-up with an extensive panel of safety measures and central laboratory testing. Like other studies in rare disorders, there were

restrictions on the number of participants that could be enrolled, which limited both group sizes and the range of AMT-060 doses that were examined. As with other similar trials, pre-treatment data was collected retrospectively, based on patient diaries that were filled out prospectively as part of routine care in these patients.[11] Also, individuals with hemophilia may inaccurately identify bleeds based on joint or muscle pain,[35] especially when chronic arthropathy and associated bone pain are present, which may have affected prospective bleed reporting.

In conclusion, a single infusion of AMT-060, is well tolerated, safe, and results in stable expression of endogenous FIX for up to 1-year of follow up. Improvement of disease severity was observed in all participants and allowed the majority (eight of nine) to discontinue FIX prophylaxis. The lack of detectable T-cell response and consequential loss of FIX activity after AAV5 gene transfer has not been observed with other AAV serotypes and is deserving of further investigation. These encouraging results support further clinical investigation of AAV5-based gene transfer for hemophilia B in a Phase 3 trial.

(German Red Cross Blood Service, Frankfurt); Ilse Tuinhof, Corine Baljé-Volkers, Tatyana De Bruyne (uniQure biopharma, B.V.)

reports a trial-related fee from UniQure. B.V. during the conduct of the study. R. Klamroth reports

grants and personal fees from Shire/Baxalta, Bayer, CSL Behring, and Pfizer outside the submitted

work; and personal fees from SOBI, Biotest, Chiesi, Octapharma, and Novo Nordisk outside the

submitted work. R. Schutgens reports financial support from UniQure B.V. paid to his institution

during the conduct of the study. At the time of contributing, M. Tangelder was a uniQure employee.

G. Castaman reports a trial-related fee from UniQure B.V. during the conduct of the study; personal

fees from Novo Nordisk, Shire, Sobi, CSL Behring, Pfizer, Bayer, outside the submitted work. J.

Schwable, B.V. H. Bonig and E. Seifried declare no conflicts of interest. F. Cattaneo is a Chiesi

Farmaceutici S.p.A employee. C. Meyer is a uniQure employee. F. W. G. Leebeek reports fees paid to

his institution from UniQure B.V. during the conduct of the study and for being a consultant for Shire

and Novo Nordisk outside the submitted work; he has received research grants from CSL Behring

and Baxalta/Shire, outside the submitted work. As part of the manuscript development process, a

one-day writing meeting was held in Amsterdam to which all authors were invited.

References

1. Fijnvandraat K, Cnossen MH, Leebeek FW, Peters M. Diagnosis and management of haemophilia. *BMJ.* 2012;344:e2707.
2. Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al. Guidelines for the management of hemophilia. *Haemophilia.* 2013;19(1):e1-47.
3. World Federation of Hemophilia. Report on the Annual Global Survey 2010. https://www1.wfh.org/publication/files/pdf-1427.pdf. Accessed 25 May 2017.
4. Rodriguez-Merchan EC. Musculoskeletal complications of hemophilia. *HSS Journal* 2010;6(1):37-42.
5. Manco-Johnson MJ, Abshire TC, Shapiro AD, et al. Prophylaxis versus episodic treatment to prevent joint disease in boys with severe hemophilia. *N Engl J Med.* 2007;357(6):535-44.
6. Santagostino E, Martinowitz U, Lissitchkov T, et al. Long-acting recombinant coagulation factor IX albumin fusion protein (rIX-FP) in hemophilia B: results of a phase 3 trial. *Blood.* 2016;127(14):1761-9.
7. Polack B, Calvez T, Chambost H, et al. EQOFIX: a combined economic and quality-of-life study of hemophilia B treatments in France. *Transfusion.* 2015;55(7):1787-97.
8. Park CY, Lee DR, Sung JJ, Kim DW. Genome-editing technologies for gene correction of hemophilia. *Hum Genet.* 2016;135(9):977-81.
9. White GC, 2nd, Rosendaal F, Aledort LM, Lusher JM, Rothschild C, Ingerslev J. Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on Thrombosis and Haemostasis. *Thromb Haemost.* 2001;85(3):560.

10. Nathwani AC, Tuddenham EG, Rangarajan S, et al. Adenovirus-associated virus vector-mediated gene transfer in hemophilia B. *N Engl J Med.* 2011;365(25):2357-65.
11. Nathwani AC, Reiss UM, Tuddenham EG, et al. Long-term safety and efficacy of factor IX gene therapy in hemophilia B. *N Engl J Med.* 2014;371(21):1994-2004.
12. Manno CS, Pierce GF, Arruda VR, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. *Nat Med.* 2006;12(3):342-7.
13. Sen D, Balakrishnan B, Gabriel N, et al. Improved adeno-associated virus (AAV) serotype 1 and 5 vectors for gene therapy. *Sci Rep.* 2013;3:1832.
14. Boutin S, Monteilhet V, Veron P, et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther.* 2010;21(6):704-12.
15. Halbert CL, Miller AD, McNamara S, et al. Prevalence of neutralizing antibodies against adeno-associated virus (AAV) types 2, 5, and 6 in cystic fibrosis and normal populations: Implications for gene therapy using AAV vectors. *Hum Gene Ther.* 2006;17(4):440-7.
16. D'Avola D, Lopez-Franco E, Sangro B, et al. Phase I open label liver-directed gene therapy clinical trial for acute intermittent porphyria. *J Hepatol.* 2016; **65**(4): 776-83.
17. EudraLex - Volume 4 - Good Manufacturing Practice (GMP) guidelines. https://ec.europa.eu/health/documents/eudralex/vol-4_en (Accessed 22 Aug 2017).
18. Lock M, McGorray S, Auricchio A, et al. Characterization of a recombinant adeno-associated virus type 2 Reference Standard Material. *Hum Gene Ther.* 2010;21(10):1273-85.
19. Ayuso E, Blouin V, Lock M, et al. Manufacturing and characterization of a recombinant adeno-associated virus type 8 reference standard material. *Hum Gene Ther.* 2014;25(11):977-87.
20. World Federation of Hemophilia. Hemophilia Joint Health Score (HJHS) 2.1.2011. http://wfh.org/2/7/7_0_Assessment_Tools_HJHS.htm. Aaccessed 25 May 2017.
21. Dimension Therapeutics I. Dimension Announces Interim Topline Results From Ongoing Phase 1/2 Clinical Program For DTX101, Dimension's Lead Aav Product Candidate In Development For Adult Patients With Moderate/Severe To Severe Hemophilia B.2017. http://investors.dimensiontx.com/phoenix.zhtml?c=254192&p=irol-newsArticle&ID=2240877. Accessed 25 May 2017.
22. George LA, Sullivan SK, Giermasz A. Adeno-Associated Virus Mediated Gene Transfer for Hemophilia B Achieves Sustained Mean Factor IX Activity Levels of >30% without Immunosuppression. *Blood.* 2016; 128: 3.
23. Ertl HC, High KA. The impact of AAV capsid-specific T cell responses on design and outcome of clinical gene transfer trials with recombinant AAV vectors - an evolving controversy. *Hum Gene Ther.* 2017.
24. Pasi J, Rangarajan S, Walsh L, et al. Interim Results from a Phase 1/2 AAV5-FVIII Gene Transfer in Patients with Severe Hemophilia A. *Res Pract Thromb Haemost.* 2017;1(Suppl. 2);1.
25. Balakrishnan B, Sen D, Hareendran S, et al. Activation of the cellular unfolded protein response by recombinant adeno-associated virus vectors. *PLoS One.* 2013; **8**(1): e53845.
26. Lange AM, Altynova ES, Nguyen GN, Sabatino DE. Overexpression of factor VIII after AAV delivery is transiently associated with cellular stress in hemophilia A mice. *Mol Ther Methods Clin Dev.* 2016; **3**: 16064.
27. Zolotukhin I, Markusic DM, Palaschak B, Hoffman BE, Srikanthan MA, Herzog RW. Potential for cellular stress response to hepatic factor VIII expression from AAV vector. *Mol Ther Methods Clin Dev.* 2016; **3**: 16063.
28. Nair N, Rincon MY, Evens H, et al. Computationally designed liver-specific transcriptional modules and hyperactive factor IX improve hepatic gene therapy. *Blood.* 2014;123(20):3195-9.
29. Crudele JM, Finn JD, Siner JI, et al. AAV liver expression of FIX-Padua prevents and eradicates FIX inhibitor without increasing thrombogenicity in hemophilia B dogs and mice. Blood. 2015;125(10):1553-61.

30. George L, Giermasz A, Sullivan S, et al. SPK-9001: Adeno-associated virus mediated gene transfer for haemophilia B achieved durable endogenous prophylaxis at levels of activity sufficient to achieve significant mean reduction in annual bleeding and infusions rates in preliminary data from an on-going Phase 1/2a trial. *Res Pract Thromb Haemost.* 2017;1(Suppl. 1);143-4.30.

31. Chapin J, Rottensteiner H, Scheiflinger F, Monahan PE. An analysis of bleeding rates and factor IX consumption in the Phase I/II BAX 335 gene therapy trial in subjects with hemophilia B. *Res Pract Thromb Haemost.* 2017;1(Suppl. 1);144-5.

32. Simioni P, Tormene D, Tognin G, et al. X-linked thrombophilia with a mutant factor IX (factor IX Padua). *N Engl J Med.* 2009;**361**(17):1671-5.

33. Monahan PE, Sun J, Gui T, et al. Employing a gain-of-function factor IX variant R338L to advance the efficacy and safety of hemophilia B human gene therapy: preclinical evaluation supporting an ongoing adeno-associated virus clinical trial. *Hum Gene Ther.* 2015;**26**(2):69-81.

34. Nathwani AC. Progress for gene therapy for hemophilia. European Society of Gene and Cell Therapy (ESGCT). Florence, Italy; 2016.

35. Ceponis A, Wong-Sefidan I, Glass CS, et al. Rapid musculoskeletal ultrasound for painful episodes in adult haemophilia patients. *Haemophilia.* 2013; **19**(5):790-8.

Tables

Variable		Cohort 1 (N=5)	Cohort 2 (N=5)
Age, years		69 (35–72)	35 (33–46)
Weight, kg		84·5 (71·2 - 89·1)	84·0 (71·4 - 96·0)
FIX Use			
Prophylaxis, IU/week[a]		4000 (2000–8000)	4000 (4000–10,500)
Annualized mean, IU/year		354,800	173,200
Mean bleeds in the year prior to enrollment,[b] n	Total	14·4	4·0
	Spontaneous	9·8	3·0
	Traumatic	2·8	1·0
	Unknown	1·8	0·0
Haemophilia joint health scores,[c]		27 (2–49)	6 (0–17)
HIV positive status, n		1	0
Prior hepatitis C infection, n		4	2

Table 1. Demographics and baseline characteristics
Values are median (min-max) unless otherwise stated. N=number
[a]1 participant in Cohort 2 received on-demand treatment and is therefore not included; [b]Historical bleed data missing for 1 participant in Cohort 2 who is therefore not included; [c]Joint status was assessed using the Haemophilia Joint Health Score version 2·1.[17] FIX, factor IX; n, number of participants with the characteristic; HIV, human immunodeficiency virus

TRAE	Number of events (number of participants) Cohort 1 (N=5)	Number of events (number of participants) Cohort 2 (N=5)
Any TRAEs	4 (3)	10 (3)
Liver enzyme increased	1 (1)	3 (2)
Pyrexia	1 (1)	2 (2)
Anxiety	1 (1)	1 (1)
Drug ineffective[a]	1 (1)	0
Palpitations	0	1 (1)
Headache	0	1 (1)
Prostatitis	0	1 (1)
Rash	0	1 (1)

Table 2. Treatment-related adverse events (TRAE) classified as possibly/probably related to treatment by the reporting investigator
TRAE were defined as AEs that were assessed by the investigator as possibly- or probably-related to AMT-060 administration. [a]As this was an early trial of gene therapy, lack of efficacy was included as an AE of special notification and was therefore automatically reported. Participant 6 in Cohort 2 experienced 7 TRAE (two liver-enzyme increases, pyrexia, anxiety, palpitations, headache and rash). TRAE, Treatment related adverse event

Participant	Pre-AMT-060 gene transfer				Post-AMT-060 gene transfer			
	FIX activity at diagnosis, IU/dL	Haemophilia B phenotype	FIX prophylaxis	Presumed cross-reactive material status[a] (activity/antigen ratio)	Mean steady state FIX, IU/dL activity (95% CI)	FIX Prophylaxis[b]	Haemophilia B phenotype	Lessening of severity
1	<1	Severe	Yes	+ (0.1)	6·2 (5·8-6·6)	No	Mild	Yes
2	<1	Severe	Yes	+ (0.4)	4·7 (4·5-5·0)	No	Moderate	Yes
3	<1	Severe	Yes	− (NC)[c]	1·3 (−0·7-3·2)	Yes	Moderate	Yes
4	1.5	Moderate	Yes	− (1.4)	6·8 (6·3-7·3)	No	Mild	Yes
5	<1	Severe	Yes	− (1.2)	3·0 (2·6-3·4)	No	Moderate	Yes
6	<1	Severe	Yes	− (1.1)	12·7 (11·9-13·5)	No	Mild	Yes
7	<1	Severe	Yes	+/− (0.7)	6·4 (6·0-6·7)	No	Mild	Yes
8	<1	Severe	No[d]	− (1.6)	6·8 (5·8-7·7)	No	Mild	Yes
9	<1	Severe	Yes	+ (0.0)	3·1 (2·8-3·3)	No	Moderate	Yes
10	<1	Severe	Yes	− (1.4)	5·8 (5·4-6·2)	No	Mild	Yes

Table 3. Mean steady state FIX levels and prophylaxis status by participant
Only values at least 10 days after the preceding FIX administration are included; [a]Baseline measurements for CRM were not collected. The majority of individuals showed post-AMT-060 activity:antigen ratios of approximately 1:1. Ratios lower than 1:1 are suggestive of CRM positivity. [d]Used on-demand FIX replacement prior to study entry. CI, confidence interval; CRM, cross-reactive material; FIX, factor IX.
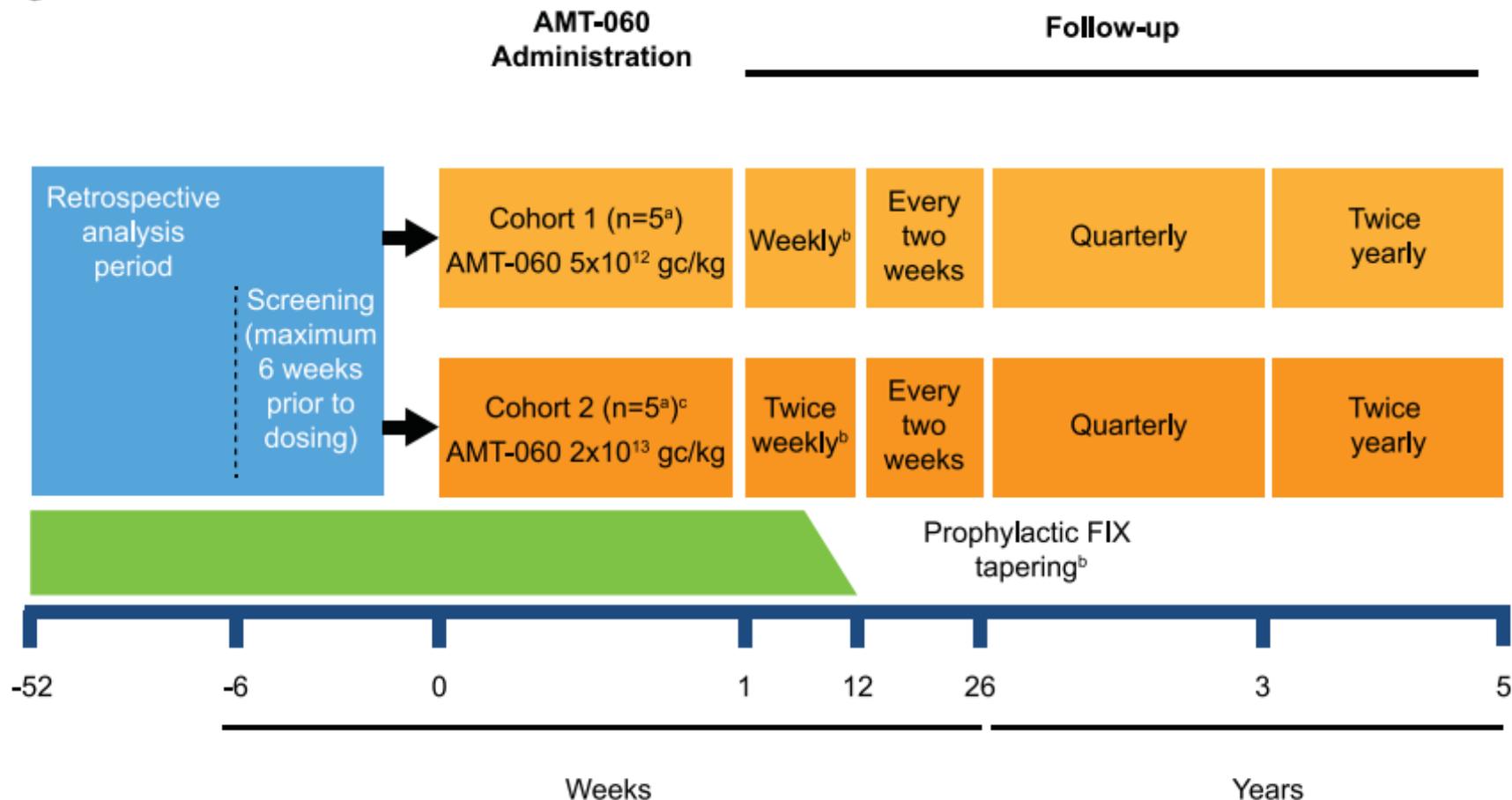
Figures

Figure 1. Study design. [a]After the administration of AMT-060 to each of the first two participants in each cohort, the Data Monitoring Committee evaluated available safety data over 24-hours before dosing of the next participant could be initiated. [b]Prophylactic FIX replacement therapy was generally tapered between weeks 6-12 if FIX activity was ≥2·0 IU/dL in at least two consecutive visits. Investigators could taper prophylaxis later than 12 weeks at their discretion. The decision to continue tapering/withholding of prophylactic FIX replacement therapy was based on the individual assessment by the investigator, but included the requirement to document that the subject could maintain a FIX activity level ≥2·0 IU/dL. [c]Cohort 2 dosing was initiated after the completion of Cohort 1 dosing and review of initial safety data by the Data Monitoring Committee. FIX, factor IX; gc, genome copies.

Figure 2. FIX activity over time. Only values at least 10 days after the preceding FIX concentrate administration, so that they are uncontaminated by exogenous FIX, are included. Participant 3 remained on prophylaxis post-AMT-060 treatment so only limited samples uncontaminated by exogenous FIX were available. The dotted line at FIX activity of 2 IU/dL indicates the threshold required for ceasing prophylaxis per protocol. FIX prophylaxis was continued after AMT-060 and tapered between Week 6 and Week 12. *Participant 4 had a moderate hemophilia B phenotype at baseline (FIX activity 1.5 IU/dL). FIX, factor IX.

Figure 3. Annualized FIX usage and bleeds. Cumulative annualized FIX activity excludes factor usage related to surgery. The total FIX usage was collected retrospectively from patient diaries and hospital record data the year before screening and prospectively post AMT-060 (excluding the tapering/prophylaxis period) as part of the statistical analysis. Follow up after discontinuation of prophylaxis ranged from 39 to 65 weeks for the participants in Cohort 1 and from 11 to 34 weeks for the participants in Cohort 2. One participant in Cohort 1 continued FIX prophylaxis following AMT-060 infusion. One participant in Cohort 2 used on demand FIX therapy prior to study entry. One participant in Cohort 2 was missing historical bleed data. FIX, factor IX; IU, international units.
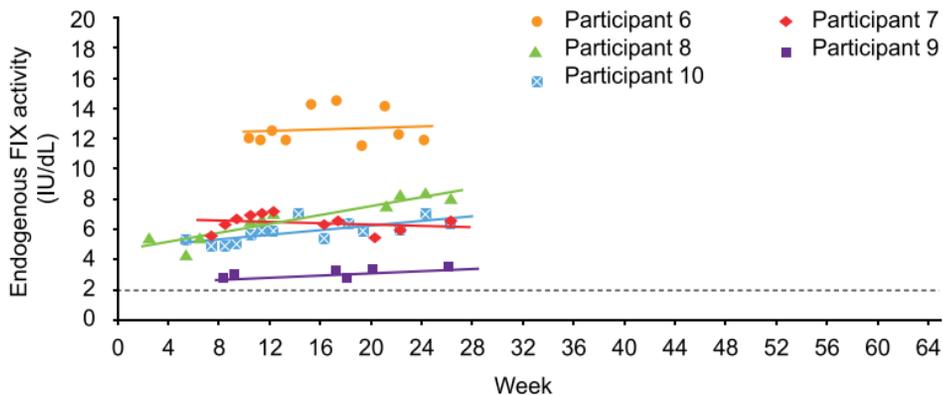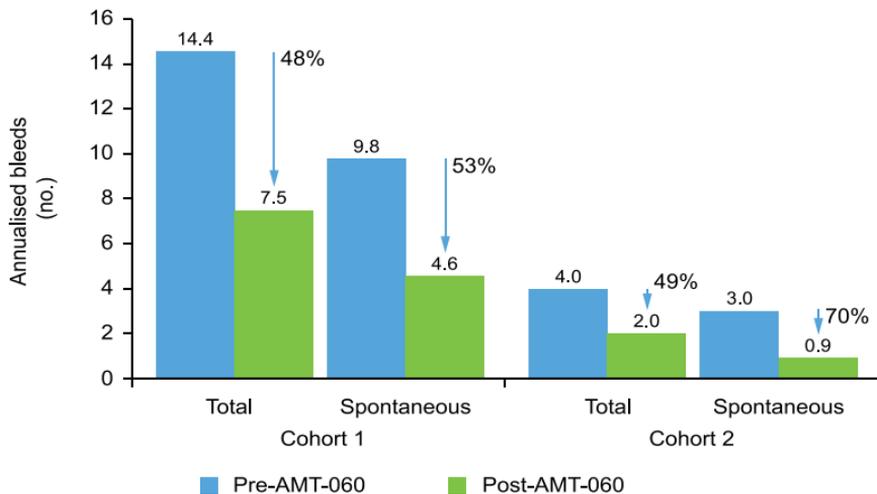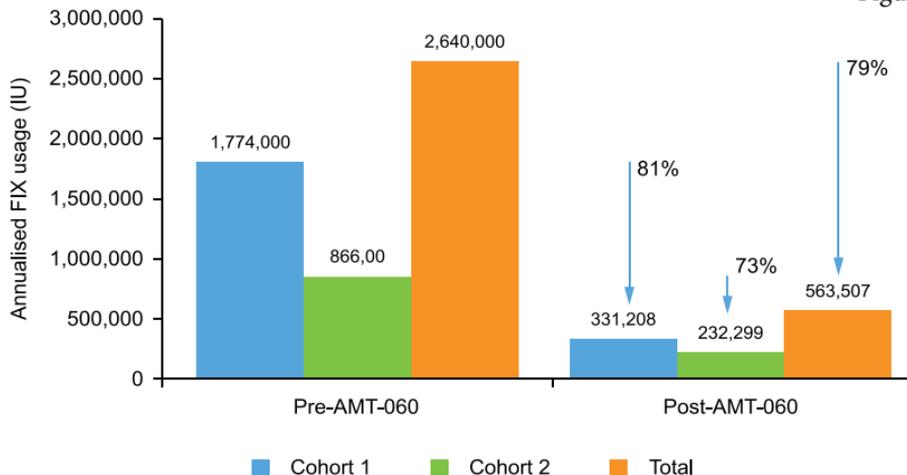
Figure 1
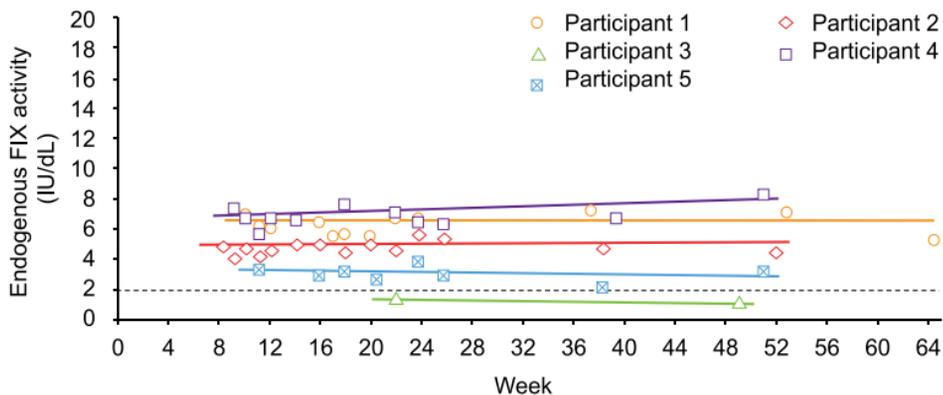
Figure 1. Study timeline showing AMT-060 Administration and Follow-up periods for Cohort 1 (n=5[a], AMT-060 5×10^{12} gc/kg) and Cohort 2 (n=5[a][c], AMT-060 2×10^{13} gc/kg).

Figure 2

A) Cohort 1

Endogenous FIX activity (IU/dL) vs Week

- Participant 1
- Participant 2
- Participant 3
- Participant 4
- Participant 5

B) Cohort 2

Endogenous FIX activity (IU/dL) vs Week

- Participant 6
- Participant 7
- Participant 8
- Participant 9
- Participant 10

Figure 3

Gene therapy with adeno-associated virus vector 5-human factor IX in adults with hemophilia B

Wolfgang Miesbach, Karina Meijer, Michiel Coppens, Peter Kampmann, Robert Klamroth, Roger Schutgens, Marco Tangelder, Giancarlo Castaman, Joachim Schwäble, Halvard Bonig, Erhard Seifried, Federica Cattaneo, Christian Meyer and Frank W.G. Leebeek