AAV5–Factor VIII Gene Transfer in Severe Hemophilia A

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ABSTRACT

BACKGROUND

Patients with hemophilia A rely on exogenous factor VIII to prevent bleeding in joints, soft tissue, and the central nervous system. Although successful gene transfer has been reported in patients with hemophilia B, the large size of the factor VIII coding region has precluded improved outcomes with gene therapy in patients with hemophilia A.

METHODS

We infused a single intravenous dose of a codon-optimized adeno-associated virus serotype 5 (AAV5) vector encoding a B-domain–deleted human factor VIII (AAV5-hFVIII-SQ) in nine men with severe hemophilia A. Participants were enrolled sequentially into one of three dose cohorts (low dose [one participant], intermediate dose [one participant], and high dose [seven participants]) and were followed through 52 weeks.

RESULTS

Factor VIII activity levels remained at 3 IU or less per deciliter in the recipients of the low or intermediate dose. In the high-dose cohort, the factor VIII activity level was more than 5 IU per deciliter between weeks 2 and 9 after gene transfer in all seven participants, and the level in six participants increased to a normal value (>50 IU per deciliter) that was maintained at 1 year after receipt of the dose. In the high-dose cohort, the median annualized bleeding rate among participants who had previously received prophylactic therapy decreased from 16 events before the study to 1 event after gene transfer, and factor VIII use for participant-reported bleeding ceased in all the participants in this cohort by week 22. The primary adverse event was an elevation in the serum alanine aminotransferase level to 1.5 times the upper limit of the normal range or less. Progression of preexisting chronic arthropathy in one participant was the only serious adverse event. No neutralizing antibodies to factor VIII were detected.

CONCLUSIONS

The infusion of AAV5-hFVIII-SQ was associated with the sustained normalization of factor VIII activity level over a period of 1 year in six of seven participants who received a high dose, with stabilization of hemostasis and a profound reduction in factor VIII use in all seven participants. In this small study, no safety events were noted, but no safety conclusions can be drawn. (Funded by BioMarin Pharmaceutical; ClinicalTrials.gov number, NCT02576795; EudraCT number, 2014-003880-38.)

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HEMOPHILIA A, AN X-LINKED BLEEDING disorder, results from mutations in the gene encoding coagulation factor VIII. Patients with severe hemophilia A (factor VIII activity level, <1 IU per deciliter) are susceptible to spontaneous or provoked bleeding in joints and soft tissue, resulting in painful disabling arthropathy, impairment in well-being, and elevated risks of intracranial hemorrhage and early death.[1,2]

Although many patients with hemophilia A receive exogenous factor VIII only when bleeding occurs (on-demand therapy), the current standard of care in developed countries is the prophylactic administration of intravenous factor VIII.[3] However, the relatively short half-life of available factor VIII and extended half-life factor VIII concentrates (8 to 19 hours) necessitates frequent infusions (up to 3 times weekly), making adherence to therapy and adequate hemostasis challenging and adversely affecting patients' quality of life.[4,5] Despite the widespread adoption of factor VIII prophylaxis in North America and Europe, breakthrough bleeding causing progressive joint destruction and impairment in quality of life still occurs,[3,6-8] particularly when trough levels of factor VIII drop below 1 IU per deciliter.[9]

Vector-mediated gene therapy has been successful in the long-term correction of underlying deficiencies in several genetic diseases.[10-12] A single infusion of an adeno-associated virus (AAV) vector expressing a human factor IX transgene led to therapeutic but low FIX plasma levels and clinical improvement for up to 3 years in 10 men with severe hemophilia B.[13,14] However, the use of AAV vectors in gene therapy for hemophilia A poses specific challenges that are due to the large size and inefficient expression of the human factor VIII coding sequence.[15-17] To overcome these obstacles, we developed AAV5-hFVIII-SQ (valoctocogene roxaparvovec), an AAV serotype 5 vector containing a codon-optimized expression cassette for the SQ variant of B-domain–deleted human factor VIII. A dose-dependent increase in factor VIII expression in mouse and nonhuman primate models after the injection of AAV5-hFVIII-SQ with a liver-specific promoter (on the basis of a construct by McIntosh et al.[17]) provided the rationale for our study. We report data from a phase 1–2 dose-escalation study of factor VIII gene transfer that used a single intravenous infusion of AAV5-hFVIII-SQ in nine men with severe hemophilia A.

METHODS

STUDY DESIGN AND ASSESSMENTS

Eligible participants were adults with severe hemophilia A, with no history of factor VIII inhibitor development and without detectable immunity to the AAV5 capsid in a cell-based in vitro transduction-inhibition assay and a total anti-AAV5 antibody assay.[18] At least 150 days of previous exposure to factor VIII concentrate or cryoprecipitate was required. For participants using on-demand factor VIII therapy, the criterion for inclusion was at least 12 bleeding episodes (defined as a bleeding event [or multiple bleeding events on same day] resulting in factor VIII replacement treatment) in the 12 months before study entry. Complete eligibility criteria are shown in the Supplementary Appendix, available with the full text of this article at NEJM.org.

From September 2015 through April 2016, nine participants were enrolled sequentially into three dose cohorts at five sites in the United Kingdom and received a single dose of AAV5-hFVIII-SQ infused (over a period of approximately 1 hour) into a peripheral vein. The low-dose cohort (one participant) received a dose of 6×10^{12} vector genomes (vg) per kilogram of body weight; the intermediate-dose cohort (one participant), 2×10^{13} vg per kilogram; and the high-dose cohort (seven participants), 6×10^{13} vg per kilogram. Escalation to the next dose cohort occurred after a single patient had received a dose safely and if the factor VIII activity level was less than 5 IU per deciliter at week 3 after gene transfer. All the participants were hospitalized for the study-drug infusion and were observed for 24 hours.

All the participants who had been receiving prophylactic factor VIII therapy previously were withdrawn from prophylaxis. However, patients were permitted to self-administer factor VIII therapy in the event of bleeding after gene transfer. On the basis of previous studies of AAV gene transfer,[13,14,19] the study protocol (available at NEJM.org) required the initiation of a therapeutic course of glucocorticoids (prednisolone at a dose of 60 mg per day, tapering over a period of ≥11 weeks) if the alanine aminotransferase level increased to 1.5 or more times the individual participant's baseline level. After the occurrence of this event in the first participant in the high-dose cohort who received the infusion (Participant 3), subsequent participants received prophylactic prednisolone (at

a dose of 40 mg per day, tapering from week 3 to week 17 or longer).

Safety was the primary end point of the study. The prospectively specified primary efficacy goal was a factor VIII activity level of at least 5 IU per deciliter at week 16 after gene transfer. Secondary efficacy measures were the frequency of factor VIII use and the number of bleeding episodes. Additional measurements included the development of factor VIII inhibitors (neutralizing antibodies), anti–AAV5 capsid total antibody levels, vector shedding, and cellular immune responses to the factor VIII transgene product and AAV capsid proteins. We are continuing to monitor long-term safety. Factor VIII activity assays were performed at a central laboratory (Esoterix) with the use of both a one-stage activated partial thromboplastin time–based clotting assay and a chromogenic factor Xa assay (see the Factor VIII Activity Level Assays section in the Supplementary Appendix).

VECTOR PRODUCTION AND FORMULATION

AAV5-hFVIII-SQ is a recombinant codon-optimized AAV5 vector that expresses the SQ variant of B-domain–deleted human factor VIII with a hybrid liver-specific transcription promoter (Fig. S1 in the Supplementary Appendix).[17,20] The expression cassette is inserted between two AAV serotype 2 inverted terminal repeats. AAV5-hFVIII-SQ was manufactured with the use of a baculovirus–*Spodoptera frugiperda* (Sf9) insect-cell production system.[21]

STUDY OVERSIGHT

The study was designed by the sponsor, BioMarin Pharmaceutical, and three authors who were not employees of the sponsor. The study was conducted in accordance with Good Clinical Practice guidelines and the principles of the Declaration of Helsinki. The study protocol was approved by relevant ethics boards. Written informed consent was provided by each participant. BioMarin Pharmaceutical oversaw the collection and analysis of data. The manuscript was written with medical writing assistance funded by BioMarin Pharmaceutical, with critical review and input from all the authors. One of the authors who is an employee of the sponsor and another employee of the sponsor performed the statistical analyses. All the authors vouch for the accuracy and completeness of the data and analyses and for the adherence of the study to the protocol.

STATISTICAL ANALYSIS

Analysis of the data was descriptive in nature, including means with standard deviations and medians, ranges, and interquartile ranges for continuous variables and counts and percentages for categorical variables. All the participants who received the study drug were included in the safety and efficacy analyses. The factor VIII activity level was assessed approximately every week at a central laboratory through week 36 and every other week through week 52 after gene transfer. Factor VIII activity levels below the lower limit of quantitation were imputed with half the lower limit of quantitation, and factor VIII activity levels obtained within 72 hours after the last use of exogenous factor VIII were excluded from the analyses. A box plot of median factor VIII activity levels within 4-week windows was generated. A regression analysis of the factor VIII activity levels in the one-stage assay and in the chromogenic assay was conducted involving participants in the high-dose cohort.

Validated medical records, diaries, and interviews with participants were used to calculate exogenous factor VIII use, factor VIII consumption, and frequency of bleeding episodes. The annualized bleeding rate for each participant was calculated as follows: (total number of bleeding episodes ÷ total number of days during the calculation period) × 365.25. Similarly, the annualized rate of factor VIII use was calculated as follows: (total number of infusions of exogenous factor VIII ÷ total number of days during the calculation period) × 365.25. The annualized factor VIII consumption was calculated as follows: (total factor VIII consumption ÷ total number of days during the calculation period) × 365.25. Adverse events were reported with the use of the Common Terminology Criteria for Adverse Events, version 4.03. A box plot of maximum alanine aminotransferase values within 4-week windows was created. The statistical analysis plan is available with the protocol.

RESULTS

CHARACTERISTICS OF THE PARTICIPANTS

Nine men with severe hemophilia A were enrolled into one of the three dose cohorts and were followed through the week 52 visit (Table 1). Eight participants had been receiving regular factor VIII prophylaxis before the study. Participant 4 used on-demand factor VIII for bleeding episodes.

SAFETY OF AAV5-HFVIII-SQ INFUSION

The adverse events that were reported in at least three of the nine participants were an increased alanine aminotransferase level (in seven), arthralgia (in six), back pain (in four), an increased aspartate aminotransferase level (in three), fatigue (in three), and productive cough (in three); all these events were of mild severity except for one moderate event of arthralgia and one moderately increased level of aspartate aminotransferase. The only serious adverse event was progression of chronic arthropathy in Participant 6, which occurred at an anatomical site that had often been a bleeding site before treatment and for which he subsequently had surgical knee replacement.

Eight participants (one in the low-dose cohort and seven in the high-dose cohort) had increased laboratory values regarding alanine aminotransferase, with the first observed value above the upper limit of the normal range at week 3; peak values ranged from 59 to 128 U per liter (normal range, 6 to 43) (Fig. 1, and Fig. S2 in the Supplementary Appendix). All these events were asymptomatic and resolved without sequelae. In two participants (Participants 1 and 5), the rise in the alanine aminotransferase level was concurrent with alcohol consumption, hepatotoxic medication (celecoxib), or vigorous exercise (or a combination of these events). No participant had abnormal elevations in the bilirubin or alkaline phosphatase level.

All the participants in the high-dose cohort received glucocorticoids (Fig. 1 and Table 1), and all had the glucocorticoids successfully tapered off. Participants 1 and 2 (in the two lower-dose cohorts) did not receive glucocorticoids during the study. There was no clear association between the resolution of the elevated alanine aminotransferase level and prednisolone use. The increased alanine aminotransferase level was accompanied by a decline in the factor VIII activity level in only one participant (Participant 4), in whom the level declined from 227 IU per deciliter to 52 IU per deciliter in conjunction with an increase in the alanine aminotransferase level from week 28 to week 35 (peak value, 95 U per liter) (Fig. 1). A decline in the alanine aminotransferase level was noted before the initiation of therapeutic prednisolone. The factor VIII activity level subsequently increased, and no bleeding was reported.

EFFICACY RESULTS

Low-Dose Cohort

In the low-dose cohort (AAV5-hFVIII-SQ at a dose of 6×10^{12} vg per kilogram), the factor VIII activity level remained under 1 IU per deciliter through week 54 in Participant 1 (Fig. 1). The consumption of factor VIII was 95 infusions per year (3792 IU per kilogram per year) before the study and 123 infusions per year (3461 IU per kilogram per year) after gene transfer. The participant resumed factor VIII prophylaxis after week 16.

Intermediate-Dose Cohort

In the intermediate-dose cohort (AAV5-hFVIII-SQ at a dose of 2×10^{13} vg per kilogram), Participant 2 had a stable but low factor VIII activity level (1 to 3 IU per deciliter) through week 54 (Fig. 1). He discontinued factor VIII prophylaxis. The consumption of factor VIII fell from 104 infusions per year before the study to 14 infusions per year after gene transfer (reduction in consumption, from 3029 to 366 IU per kilogram per year), but the annualized bleeding rate increased from 3 to 11 events. The participant elected to continue with on-demand therapy.

High-Dose Cohort

After the infusion in the high-dose cohort (AAV5-hFVIII-SQ at a dose of 6×10^{13} vg per kilogram), the factor VIII activity level gradually increased and then appeared to plateau at or above physiologic levels in weeks 20 through 24 (Figs. 1 and 2). At week 16 (the prespecified time point for the efficacy assessment), all seven participants had a factor VIII activity level that was more than 5 IU per deciliter (5 IU per deciliter is the cutoff for moderate vs. mild hemophilia); this level occurred in four participants by week 2 (Table 1). After week 20, the factor VIII activity level was consistently more than 50 IU per deciliter in six of seven participants, and in the remaining participant the level typically ranged from 12 to 32 IU per deciliter. At week 52, the median factor VIII activity level was 77 IU per deciliter (range, 19 to 164; mean [±SD] level, 93±48). The factor VIII activity levels reported here are from the one-stage clotting assay and were consistently approximately 1.65 times as high as the factor VIII levels from the chromogenic assay (see the Comparison of One-Stage and Chromogenic Assays

Table 1. Characteristics of the Participants at Baseline and after Gene Transfer, According to Dose Cohort.*

| Characteristic	Low Dose	Intermediate Dose	High Dose						
	Participant 1	Participant 2	Participant 3	Participant 4	Participant 5	Participant 6	Participant 7	Participant 8	Participant 9
At baseline									
Age (yr)	25	43	32	23	28	30	30	28	42
Race†	Asian	White	White	Asian	White	White	White	White	White
Weight (kg)	71	103	89	60	70	81	86	72	77
Genetic mutation	Intron 22 inversion	Intron 22 inversion	Intron 22 inversion	Single nucleotide duplication	2-bp deletion	Nonsense mutation (A2)	Missense mutation (A2)	Splice-site mutation (intron 2)	Single nucleotide duplication
Factor VIII use									
Type of replacement therapy	Prophylactic	Prophylactic	Prophylactic	On demand	Prophylactic	Prophylactic	Prophylactic	Prophylactic	Prophylactic
Consumption in previous year (IU/kg/yr)	3792	3029	4218	833‡	5460	5903	3634	5112	6373
Annualized bleeding rate in year before enrollment (no. of events)	2	3	9	—§	1	24	40	24	0
ALT (U/liter)	24	22	12	16	32	17	45	28	26
History of HCV infection	Negative	Positive, cleared	Negative	Negative	Negative	Negative	Positive, cleared	Negative	Positive, cleared
After gene transfer									
Factor VIII activity (IU/dl)¶									
At 20 wk	NA	2	72	219	237	12	142	36	109
At 52 wk	NA	2	100	76	164	19	141	77	77
Time until factor VIII activity level >5 IU/dl (wk)¶	NA	NA	4	2	6	9	2	2	2
Annualized bleeding rate after factor VIII activity level >5 IU/dl (no. of events)¶	NA	NA	0	1	0	3.5	0	0	0
Total factor VIII consumption (IU/kg/yr)	3461	366	33	81	139	676	89	40	0
Peak ALT (U/liter)	128	33	60	95	82	87	59	81	66
Total duration of glucocorticoid use (wk)	0	0	23	26	17	32	13	24	14

* The low-dose cohort included one participant, who received 6×10^{12} vector genomes (vg) per kilogram of body weight; the intermediate-dose cohort included one participant, who received 2×10^{13} vg per kilogram; and the high-dose cohort included seven participants, who received 6×10^{13} vg per kilogram. HCV denotes hepatitis C virus, and NA not applicable.
† Race was reported by the participant and recorded in his medical chart. The normal range for the alanine aminotransferase (ALT) level is 6 to 43 U per liter.
‡ The value for Participant 4 was calculated from prescription-utilization data.
§ The value was not available for Participant 4.
¶ Factor VIII values are from the one-stage assay performed by a central laboratory (Esoterix); the normal range is 50 to 150 IU per deciliter. Week 20 values are reported here as being representative of the time point after which factor VIII activity levels were approximately stable in all participants. Participant 1 did not have data for factor VIII activity available within a 72-hour period since the last consumption of exogenous factor VIII.
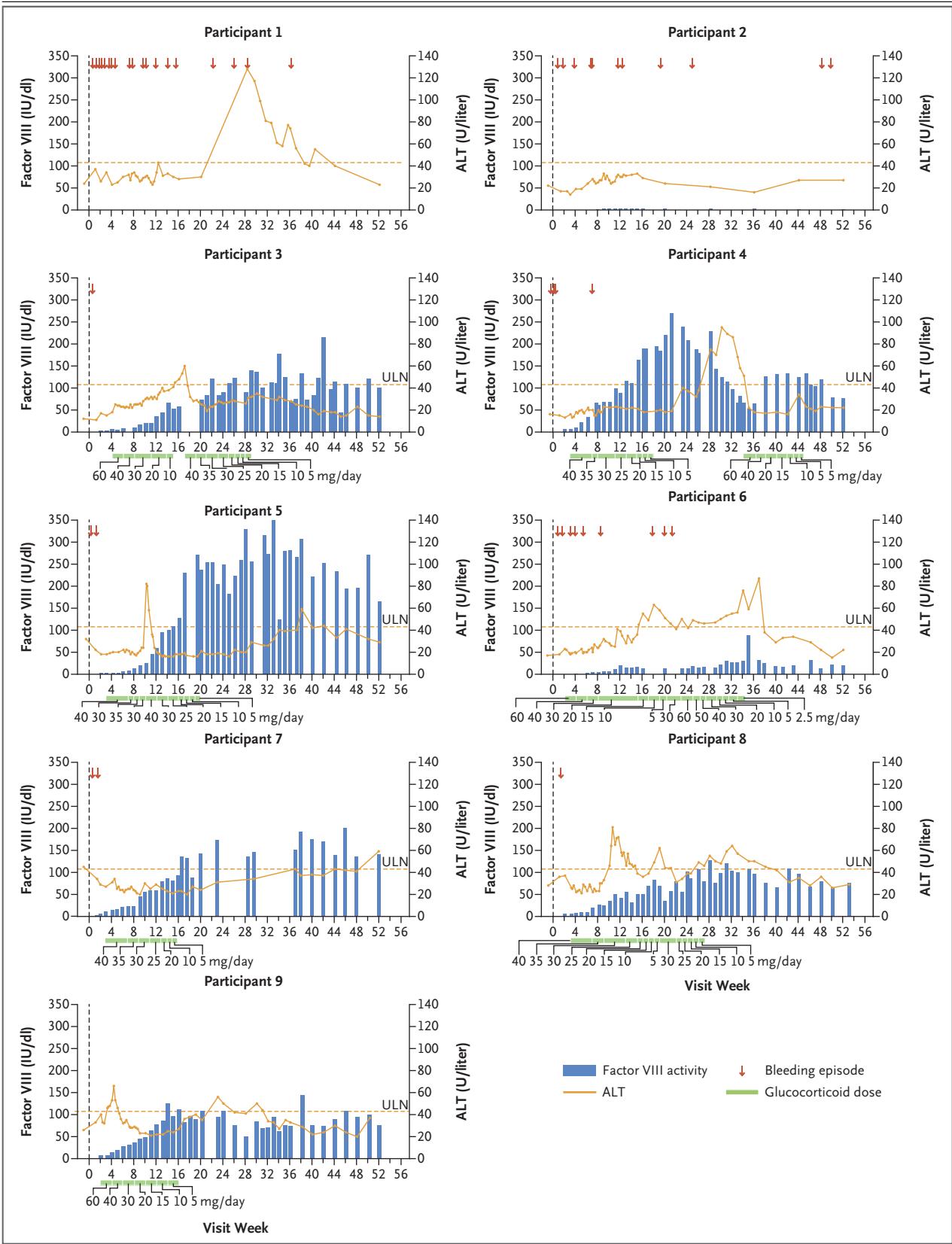
Figure 1 (facing page). Individual Participant Profiles, Including Factor VIII Activity Levels, Bleeding Episodes, Levels of Alanine Aminotransferase, and Glucocorticoid Use.

Participant 1 was in the low-dose cohort (dose, 6×10^{12} vector genomes [vg] per kilogram of body weight), Participant 2 in the intermediate-dose cohort (dose, 2×10^{13} vg per kilogram), and Participants 3 through 9 in the high-dose cohort (dose, 6×10^{13} vg per kilogram). The dose (dashed vertical line) was administered on day 0. Factor VIII values are from the one-stage assay performed by a central laboratory (Esoterix); the normal range is 50 to 150 IU per deciliter. The normal range for the alanine aminotransferase (ALT) level is 6 to 43 U per liter; the upper limit of the normal range is indicated by the horizontal dashed line. Arrows indicate bleeding episodes that were reported by the participant. The dose of glucocorticoids (values associated with green bars) in Participants 3 through 9 is shown in milligrams per day; Participants 1 and 2 did not use glucocorticoids.

for Factor VIII Activity section and Fig. S3 in the Supplementary Appendix).

In the six participants who had received factor VIII prophylaxis before the study, the median annualized bleeding rate dropped from 16 events per year before the study to 1 event per year after gene transfer (mean reduction in rate, from 16 to 2 events) (Fig. 3A). The median annualized use of factor VIII fell from 138 infusions per year before the study to 2 infusions per year after gene transfer (mean reduction in rate, from 137 to 5 infusions per year) and was 0 after week 2 (i.e., when the endogenous factor VIII activity level reached 1 to 5 IU per deciliter) (Fig. 3B). The median consumption of factor VIII decreased from 5286 to 65 IU per kilogram per year. One participant did not use factor VIII at all after gene transfer, and four ceased factor VIII use after week 2. Only Participant 6 used factor VIII for self-reported bleeding after week 2 — at week 21 for a knee that had undergone multiple radiosynovectomy procedures previously; at this time, his circulating factor VIII level was 12 IU per deciliter. He subsequently underwent total knee replacement, with perioperative use of factor VIII, at week 54.

In the one participant (Participant 4) who had previously used on-demand factor VIII therapy, factor VIII consumption fell from 833 IU per kilogram per year before the study to 81 IU per kilogram per year after gene transfer. After week 2, the participant used factor VIII only once for

self-diagnosed bleeding at week 7; the factor VIII activity level at the time was 34 to 66 IU per deciliter.
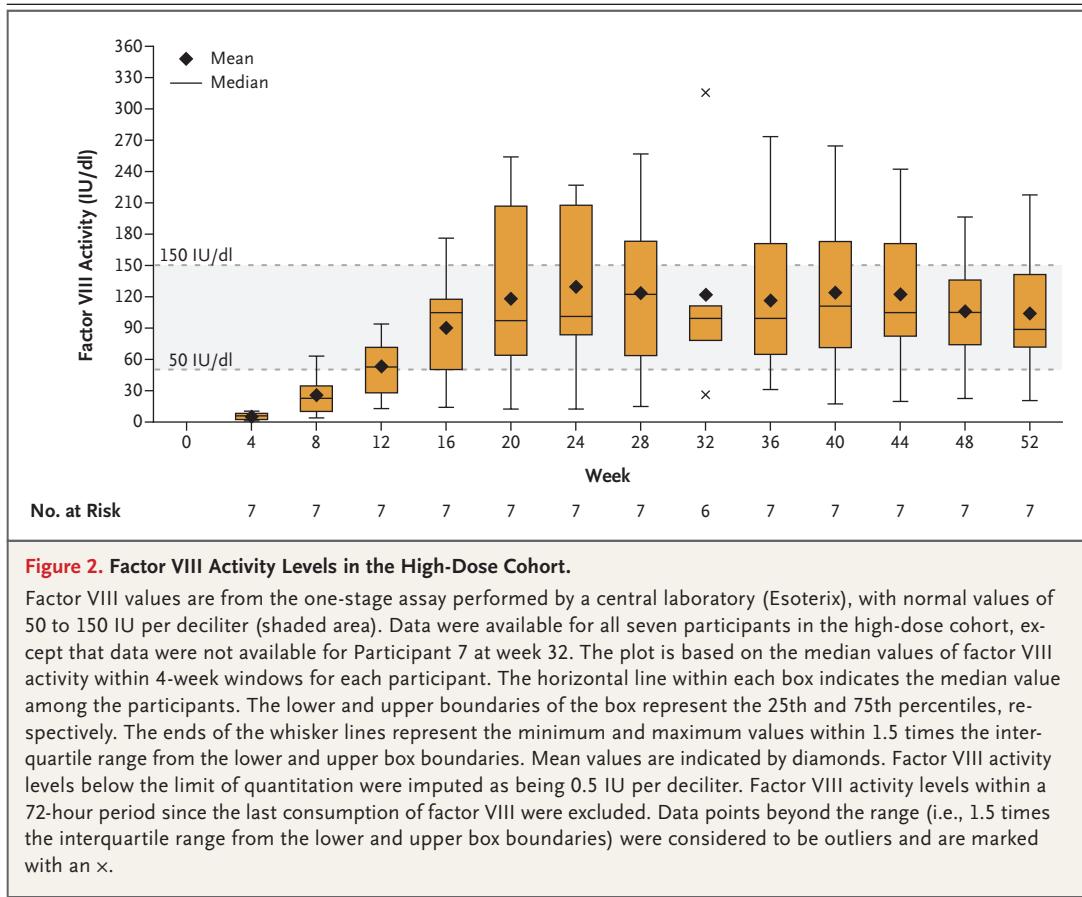
HOST IMMUNE RESPONSE TO FACTOR VIII AND AAV5

AAV5 capsid-specific antibodies developed after gene transfer in all the participants by week 8 (the first time point assessed). However, cellular immune responses that were specific for AAV5 capsid peptides were not detected at any time point, according to interferon-γ enzyme-linked immunospot (ELISPOT) assay. In Participants 6 and 9, at one time point each, the results on the interferon-γ ELISPOT assay above the established threshold for positivity (50 spot-forming units [SFU] per 10^6 peripheral-blood mononuclear cells) were detected against FVIII-SQ peptides. These responses, with a maximal value of 120 SFU per 10^6 peripheral-blood mononuclear cells, were not consistently associated with increasing levels of alanine aminotransferase or declines in measures of factor VIII and returned to negative 4 weeks later. No participant tested positive for factor VIII inhibitors at any time point according to the Nijmegen–Bethesda assay.

VECTOR SHEDDING

Vector DNA was detected by the quantitative polymerase-chain-reaction (PCR) assay in blood, semen, saliva, urine, and feces within 72 hours after infusion in all the participants, with peak levels in weeks 1 through 4. Samples were reported as negative only if no signal on the quantitative PCR assay above the threshold of amplification was observed; otherwise, the results were reported as positive. Positive samples at or above the limit of quantitation were reported with numerical results, and positive samples below the limit of quantitation were reported as being below the limit of quantitation. Clearance for each matrix was defined as having had negative results at three consecutive visits.

Overall, all the samples of biologic fluids and feces showed decreasing quantities of residual vector DNA over the study period. For the two participants in the two lower-dose cohorts, the fastest clearing biologic fluids were urine (5 weeks and 11 weeks) and semen (11 weeks and 13 weeks). Each of these participants had two consecutive negative results in saliva at week 52. The samples from Participant 1 (in the low-dose cohort) indicated that feces was cleared and the blood

Figure 2. Factor VIII Activity Levels in the High-Dose Cohort.

Factor VIII values are from the one-stage assay performed by a central laboratory (Esoterix), with normal values of 50 to 150 IU per deciliter (shaded area). Data were available for all seven participants in the high-dose cohort, except that data were not available for Participant 7 at week 32. The plot is based on the median values of factor VIII activity within 4-week windows for each participant. The horizontal line within each box indicates the median value among the participants. The lower and upper boundaries of the box represent the 25th and 75th percentiles, respectively. The ends of the whisker lines represent the minimum and maximum values within 1.5 times the interquartile range from the lower and upper box boundaries. Mean values are indicated by diamonds. Factor VIII activity levels below the limit of quantitation were imputed as being 0.5 IU per deciliter. Factor VIII activity levels within a 72-hour period since the last consumption of factor VIII were excluded. Data points beyond the range (i.e., 1.5 times the interquartile range from the lower and upper box boundaries) were considered to be outliers and are marked with an ×.

level was below the limit of quantitation at week 52. The samples from Participant 2 (in the intermediate-dose cohort) indicated one negative result in the feces compartment and a blood level above the limit of quantitation at week 52.

In the high-dose cohort, residual levels of vector DNA were present in all seven participants at week 52 in blood, with all values above the limit of quantitation. The fastest clearing biologic fluid was urine, with all participants having urine cleared at or before 28 weeks (range, 6 to 28) (Fig. 4). Four of the seven participants had semen cleared at or before 36 weeks (range, 16 to 36); of the remaining three participants, one had two consecutive negative results (this participant had semen cleared at week 56 when three consecutive negative results were obtained), and two had levels below the limit of quantitation in semen at week 52. On further investigation, we found that vector DNA was not present in purified sperm cells that were obtained from these two participants, which ruled out the risk of

inadvertent germline modification. All the participants in the high-dose cohort had samples showing that saliva was cleared at or before 52 weeks (range, 40 to 52). No participant in the high-dose cohort had a sample showing that feces was cleared at week 52, but all the levels were below the limit of quantitation. The household contacts of the participants were not examined.

DISCUSSION

We report the results of a phase 1–2, dose-escalation study to assess the safety and efficacy of a single peripheral infusion of AAV5-hFVIII-SQ, a codon-optimized AAV5 vector encoding B-domain–deleted human factor VIII, in nine men with severe hemophilia A. These changes in the vector and the gene resulted in successful gene transfer in participants with hemophilia A, despite the large size of the coding region.
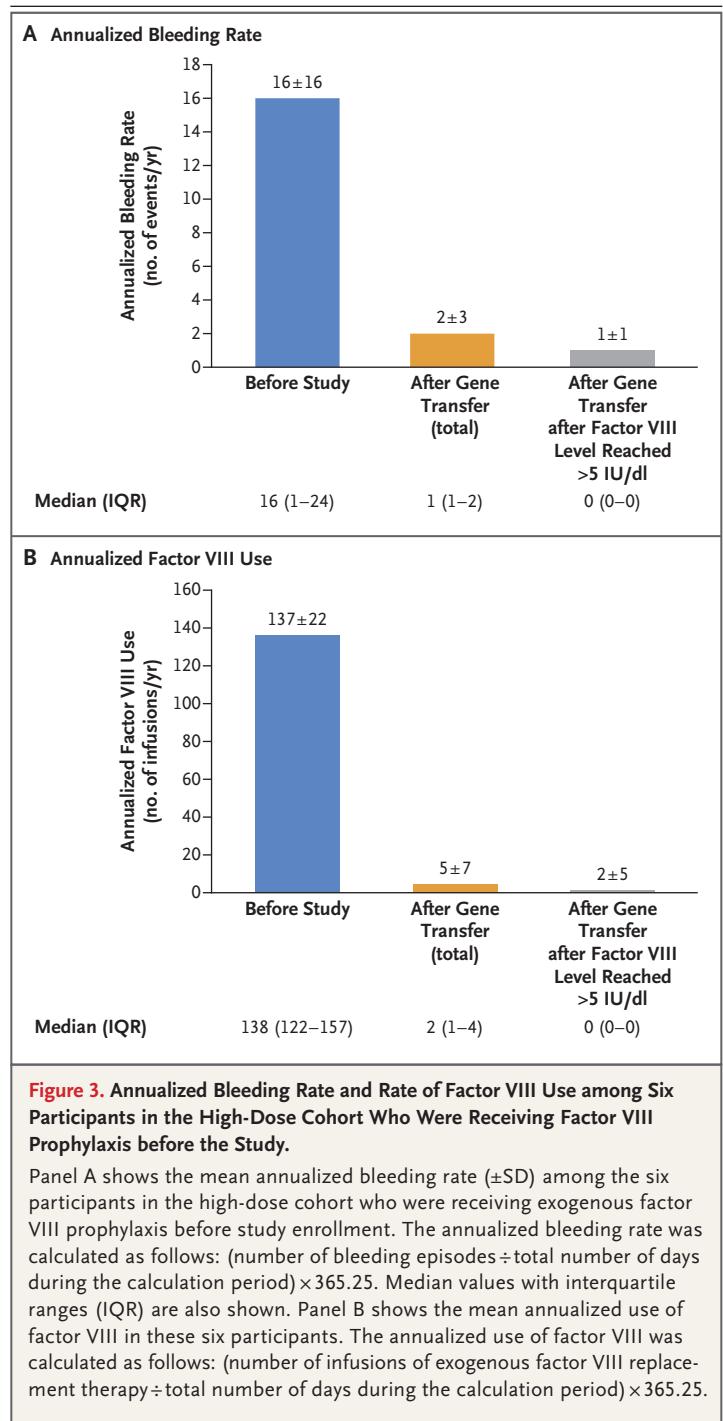
Increases in the factor VIII activity levels were dose-dependent, with all seven participants in

the high-dose cohort (dose, 6×10^{13} vg per kilogram) sustaining therapeutic levels at 1 year after gene transfer. In conjunction, the frequency of participant-treated bleeding episodes decreased markedly, with resultant cessation of factor VIII use. The absence of spontaneous bleeding in patients with severe hemophilia A is uncommon with factor VIII replacement therapy, regardless of the product half-life. Cessation of prophylactic infusions, reduced use of pain medications and other medications, freedom from microbleeding, and absence of unprotected periods of factor VIII trough levels of less than 1 IU per deciliter from replacement therapy are likely to provide improved quality of life to patients.

Three previous clinical trials of gene transfer in patients with severe hemophilia A have been unsuccessful. In two trials, one with the use of ex vivo transfected dermal fibroblasts expressing a modified factor VIII molecule[22] and one with the use of a retroviral vector expressing factor VIII,[23] the sustained expression of adequate factor VIII levels did not occur. A third trial, in which adenovirus was used to deliver the factor VIII gene, was terminated owing to toxic events in a single patient.[24]

In contrast, in the current study, six of seven participants in the high-dose cohort had a factor VIII activity level in or above the physiologic range (50 to 150 IU per deciliter) at 1 year after gene transfer (range, 76 to 164 IU per deciliter), and the factor VIII activity level in the remaining participant was within the range for mild hemophilia (5 to 40 IU per deciliter). A factor VIII activity level of more than 5 IU per deciliter was reached in all seven participants between weeks 2 and 9 after gene transfer, with stabilization between weeks 20 and 24. Observations of prolonged clinical benefit correspond with preclinical findings showing the persistence of factor VIII expression for multiple years after AAV gene therapy in dogs.[15] In view of studies that document a close association between the factor VIII activity level and bleeding frequency (i.e., an 18% reduction with every increase of 1 IU per deciliter in the factor VIII activity level),[25] as well as between total and joint hemorrhages and disabling arthropathy,[8] the achievement of sustained factor VIII activity levels of more than 5 IU per deciliter after gene transfer in all the recipients in the high-dose cohort is encouraging.

The variation in the factor VIII activity levels

A Annualized Bleeding Rate

	Before Study	After Gene Transfer (total)	After Gene Transfer after Factor VIII Level Reached >5 IU/dl
Mean ±SD	16±16	2±3	1±1
Median (IQR)	16 (1–24)	1 (1–2)	0 (0–0)

B Annualized Factor VIII Use

	Before Study	After Gene Transfer (total)	After Gene Transfer after Factor VIII Level Reached >5 IU/dl
Mean ±SD	137±22	5±7	2±5
Median (IQR)	138 (122–157)	2 (1–4)	0 (0–0)

Figure 3. Annualized Bleeding Rate and Rate of Factor VIII Use among Six Participants in the High-Dose Cohort Who Were Receiving Factor VIII Prophylaxis before the Study.

Panel A shows the mean annualized bleeding rate (±SD) among the six participants in the high-dose cohort who were receiving exogenous factor VIII prophylaxis before study enrollment. The annualized bleeding rate was calculated as follows: (number of bleeding episodes ÷ total number of days during the calculation period) × 365.25. Median values with interquartile ranges (IQR) are also shown. Panel B shows the mean annualized use of factor VIII in these six participants. The annualized use of factor VIII was calculated as follows: (number of infusions of exogenous factor VIII replacement therapy ÷ total number of days during the calculation period) × 365.25.

among the participants is likely to be multifactorial and may stem in part from differences in vector uptake within cells and in the synthesis, release, and metabolism of the factor VIII protein in the circulation. AAV5 delivery of the factor VIII transgene appears to take longer to reach
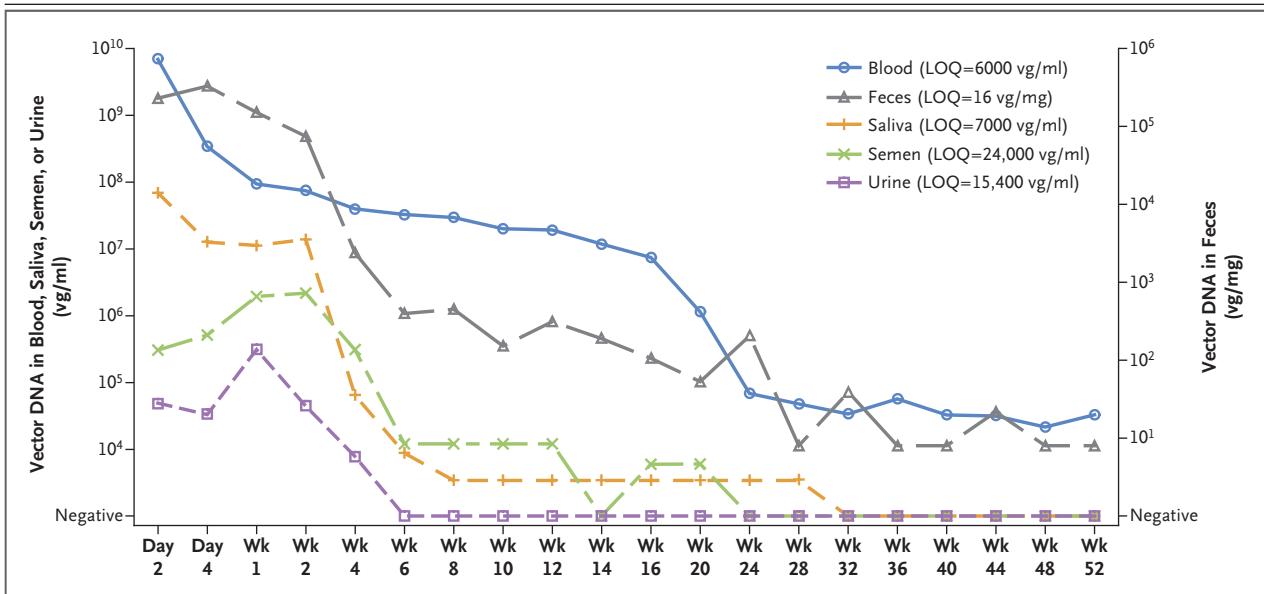
Figure 4. Median Levels of Vector DNA in Biologic Fluids in the High-Dose Cohort.

The plot is based on the median level of vector DNA at each visit. When multiple test results were available for a participant in a visit window, the maximum result was picked for the participant for the median calculation of that visit. Negative values were carried over to the following visits without additional testing, after three consecutive negative test results were observed (i.e., after clearance was observed). The data-cutoff date was based on the 52-week observation window. Values below the limit of quantitation (LOQ) were imputed as one half the validated LOQ of 50 vg per quantitative polymerase chain reaction and were then back-calculated to the theoretically corresponding vector genomes per standard unit of biologic specimen.

a steady state than has been observed in previous studies that have used other serotypes and the *FIX* transgene; different expression kinetics between AAV serotypes[26] and slow annealing of partial single-stranded DNA molecules because of the size of the factor VIII transgene may contribute to this difference.

As in other trials of AAV-based gene therapy,[13,19,27,28] a mild asymptomatic increase in the serum level of alanine aminotransferase was common. In contrast to results in a trial of an AAV serotype 8 vector in patients with hemophilia B,[13,14] only one participant in our study had a decline in the factor VIII activity level with an increased level of alanine aminotransferase. The continued increase in factor VIII activity level concurrent with the increased level of alanine aminotransferase in the other six participants in the high-dose cohort, as well as the absence of a concomitant rise in the bilirubin or alkaline phosphatase level, is consistent with maintenance of a high level of hepatocyte function and suggests that hepatic toxicity may not be a limiting factor in treatment.

After infusion, antibodies to AAV5 were detected in all the tested participants, but no T-cell–mediated immune responses to AAV5 capsid proteins were detected, and neutralizing antibodies to factor VIII did not develop in any participants. The absence of factor VIII inhibitors during more than 1 year of follow-up underscores the safety of AAV5-hFVIII-SQ. As has been observed in similar clinical trials of vector gene transfer,[13,19,29] vector DNA was detected in various biologic fluids obtained from all the participants (see the Vector Shedding–Discussion section in the Supplementary Appendix). The possibility of vector integration was not assessed. The protocol was not designed to measure whether AAV5 infection was present in family members or close contacts, and ethics approval was not sought to pursue this question. The two viral genes within AAV5 have been replaced by factor VIII, and AAV5 requires another virus such as an adenovirus for a productive infection.

Factor VIII activity levels of more than 150 IU per deciliter were observed intermittently in four participants in the high-dose cohort, with peak

values ranging from 201 to 349 IU per deciliter. These values were not associated with clinical findings that were suggestive of a thrombotic event and did not lead to medical intervention. Periodic assessments of the platelet count, prothrombin time, and activated partial-thromboplastin time were within normal limits in these participants. Preliminary data regarding six participants who received a dose (4×10^{13} vg per kilogram) between the intermediate and high doses in our study support a dose response, with maximum factor VIII activity levels between 3 and 50 IU per deciliter (during 5 to 20 weeks of observation).

Although mild asymptomatic elevations in the serum level of alanine aminotransferase were common, these were usually not associated with decreased factor VIII activity levels and were without clinical sequelae. We observed sustained factor VIII activity at therapeutic levels for 1 year in seven men with severe hemophilia A after a single intravenous infusion of a codon-optimized AAV5 vector expressing the SQ variant of the B-domain–deleted human factor VIII (at a dose of 6×10^{13} vg per kilogram). Increased levels of factor VIII activity were accompanied by a marked diminution in the rate of bleeding episodes and the cessation of exogenous factor VIII use.

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