Multiyear Follow-up of AAV5-hFVIII-SQ Gene Therapy for Hemophilia A

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ABSTRACT

BACKGROUND

Adeno-associated virus (AAV)–mediated gene therapy is under investigation as a therapeutic option for persons with hemophilia A. Efficacy and safety data include 3 years of follow-up after a single administration of AAV5-hFVIII-SQ.

METHODS

We report durable efficacy, long-term safety, and clinical and biologic results in 15 adults with severe hemophilia A (factor VIII level, ≤1 IU per deciliter) who had received a single infusion of AAV5-hFVIII-SQ at various dose levels. We evaluated the factor VIII level, annualized rate of bleeding events, use of factor VIII, safety, expression kinetics, and biologic markers of AAV transduction for up to 3 years.

RESULTS

Three years after infusion, two participants (one who had received 6×10^{12} vector genomes [vg] per kilogram of body weight and one who had received 2×10^{13} vg per kilogram) had factor VIII expression of less than 1 IU per deciliter, as assessed on chromogenic assay. Seven participants (who had received 6×10^{13} vg per kilogram) had a median factor VIII expression of 20 IU per deciliter; the median number of annualized treated bleeding events was 0, and the median use of exogenous factor VIII was reduced from 138.5 infusions to 0 infusions per year. Bleeding in all target joints (major joints with ≥3 bleeding events within 6 months) in this cohort resolved (≤2 bleeding events within 12 months). Two years after infusion, six participants (who had received 4×10^{13} vg per kilogram) had a median factor VIII expression of 13 IU per deciliter; the median annualized rate of bleeding events was 0, and the median use of factor VIII was reduced from 155.5 infusions to 0.5 infusions per year. Bleeding in target joints resolved in five of six participants. The factor VIII pharmacodynamic profiles reflected cellular turnover in the blood and molecular events leading to episomal DNA stabilization for persistent expression, findings that are consistent with previous observations in two model systems. Transgene-derived human factor VIII (hFVIII) protein activity mirrored native hFVIII in hemostatic ability. No inhibitor development, thromboses, deaths, or persistent changes in liver-function tests were observed.

CONCLUSIONS

Gene therapy with AAV5-hFVIII-SQ vector in participants with hemophilia A resulted in sustained, clinically relevant benefit, as measured by a substantial reduction in annualized rates of bleeding events and complete cessation of prophylactic factor VIII use in all participants who had received 4×10^{13} vg per kilogram or 6×10^{13} vg per kilogram of the gene therapy. (Funded by BioMarin Pharmaceutical; ClinicalTrials.gov number, NCT02576795; EudraCT number, 2014-003880-38.)

ADENO-ASSOCIATED VIRUS (AAV)–MEDI-ated gene therapy is increasingly recognized for its potential to treat many monogenic diseases, including hemophilia A and B, by means of delivery of complementary DNA encoding functional factor VIII or factor IX proteins, respectively. To date, a single administration of AAV-mediated investigational therapy has shown clinical improvements for 1 year with the use of an AAV serotype 5 vector (AAV5) for hemophilia A.[1]

Here, we describe 2-year and 3-year safety and efficacy data after the administration of AAV5-hFVIII-SQ (valoctocogene roxaparvovec) in men with severe hemophilia A. In addition to providing clinical safety and efficacy data, this research contributes to a mechanistic understanding of the variability observed in gene therapy studies,[2-6] thus providing insights into mechanisms of DNA persistence and durable expression.

METHODS

VECTOR CONSTRUCT

Owing to the large size of the factor VIII gene relative to the packaging capacity of AAV of approximately 4.7 kb,[7] the AAV5-hFVIII-SQ expression cassette contains a B-domain–deleted (SQ variant) human factor VIII transgene and a small efficient promoter,[8] manufactured as described previously.[1] At approximately 5 kb, the condensed expression cassette is incompletely packaged into each AAV capsid and requires a complementary vector particle for annealing to form full-length, expression-competent, double-stranded DNA episomes in cell nuclei (Fig. S3 in the Supplementary Appendix, available with the full text of this article at NEJM.org).[9]

STUDY DESIGN AND ASSESSMENTS

A phase 1–2 dose-escalation, safety, and efficacy study was initiated in June 2015 as described previously[1]; the protocol (including the statistical analysis plan) is available at NEJM.org. The study was designed by the sponsor, BioMarin Pharmaceutical, with input from the authors, who vouch for the accuracy and completeness of the data and for the adherence of the study to the protocol. Writing assistance was provided by the sponsor.

A total of 15 adult male participants with severe hemophilia A were enrolled. The cutoff date for all data reported here was April 1, 2019.

The 14 participants who were using prophylactic factor VIII replacement therapy were required to discontinue prophylactic infusions at the time of study enrollment, but participants were permitted to administer factor VIII therapy themselves in the event of a bleeding episode during the study. All the participants were hospitalized and observed for 24 hours after the infusion of AAV5-hFVIII-SQ at the following doses: cohort 1 included Participant 1, who received 6×10^{12} vector genomes (vg) per kilogram of body weight; cohort 2 included Participant 2, who received 2×10^{13} vg per kilogram; cohort 3 included Participants 3 through 9, who received 6×10^{13} vg per kilogram; and cohort 4 included Participants 10 through 15, who received 4×10^{13} vg per kilogram. Dose escalation was monitored, such that the administration of the doses in each participant occurred only after requisite safety checks in previous participants, as described previously.[1]

As specified in the protocol, prophylactic glucocorticoids were to be administered in all subsequent participants if the alanine aminotransferase level after infusion reached 1.5 times the baseline value in any participant who received a dose. This occurred in Participant 3, and so all remaining six participants in the cohort that received 6×10^{13} vg per kilogram (cohort 3) received prophylactic glucocorticoids at a dose of 40 mg per day, starting 3 weeks after infusion; the dose was then tapered. Participants received glucocorticoid therapy for at least 15 weeks. After an amendment to the protocol, the requirement for glucocorticoid prophylaxis was removed, so participants who received 4×10^{13} vg per kilogram (cohort 4) were given glucocorticoids, at an initial dose of 60 mg per day, only in response to an elevation in the alanine aminotransferase level.

RESULTS

LONG-TERM SAFETY OF AAV5-hFVIII-SQ

The characteristics of the participants are described in Table 1 and in Table S1. All the participants had at least one adverse event (Tables S2 and S3), and no participants withdrew from the study. The most common adverse event was an elevation of the alanine aminotransferase level, with 14 reported events (13 events of grade 1

Table 1. Characteristics of Participants 3 through 15 at Baseline and in the Years after Gene Transfer.*

Characteristic	Cohort 3							Cohort 4					
	Pt 3	Pt 4	Pt 5	Pt 6	Pt 7	Pt 8	Pt 9	Pt 10	Pt 11	Pt 12	Pt 13	Pt 14	Pt 15
At baseline													
Age (yr)	32	23	28	30	30	28	42	24	37	22	23	45	37
Factor VIII use													
Prophylactic replacement therapy	Yes	No†	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Use in previous year (IU/kg)	4218	833‡	5460	5903	3634	5112	6373	6325	4148	3099	1709	5763	7661
Annualized rate of bleeding events in previous year§	9	NA	1	24	40	24	0	1	41	0	4	15	12
After gene transfer													
Year 1													
Annualized rate of bleeding events (no. of events)§	0.0	1.1	0.0	5.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.4
Annualized factor VIII use (no. of infusions)¶	0	1	0	12	0	0	0	0	0	0	1	0	10
Factor VIII activity (IU/dl)‖	62	50	95	11	88	52	55	18	40	24	14	26	<3
Year 2													
Annualized rate of bleeding events (no. of events)§	0.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	6.0
Annualized factor VIII use (no. of infusions)¶	1	0	0	52	0	0	0	1	0	0	0	1	39
Factor VIII activity (IU/dl)‖	26	25	38	4	86	24	52	13	20	10	8	22	6
Year 3**													
Annualized rate of bleeding events (no. of events)§	0.0	0.0	0.0	4.0	0.0	0.0	0.0	NA	NA	NA	NA	NA	NA
Annualized factor VIII use (no. of infusions)¶	23	1	0	10	0	0	0	NA	NA	NA	NA	NA	NA
Factor VIII activity (IU/dl)‖	11	20	31	4	100	17	36	NA	NA	NA	NA	NA	NA

* Participants 3 through 9 (cohort 3) received 6×10^{13} vector genomes (vg) per kilogram of body weight, and Participants 10 through 15 (cohort 4) received 4×10^{13} vg per kilogram. The demographic and clinical characteristics of the two participants in cohorts 1 and 2 (in which a single participant received 6×10^{12} vg per kilogram and 2×10^{13} vg per kilogram, respectively) are shown in Table S1. NA denotes not applicable, and Pt participant.

† Participant 4 used on-demand factor VIII replacement therapy.

‡ The value was calculated from prescription utilization.

§ The annualized rate of bleeding events was calculated as the total number of bleeding events within a period, divided by the duration of that period (in days), multiplied by 365.25.

¶ Annualized factor VIII use was calculated as the total number of factor VIII infusions within a period, divided by the duration of that period (in days), multiplied by 365.25.

‖ Factor VIII activity was assessed on chromogenic assay; the normal range is 50 to 150 IU per deciliter.

** The factor VIII assessments at year 3 were collected at week 156 with a window of ±4 weeks, for a range of collection dates from week 152 to 160.

and 1 event of grade 2) in 11 participants. Individual elevations of the alanine aminotransferase level are detailed in Figure 1 and in Figure S1. Two elevations were assessed by the investigator as being not related to treatment, and all the events were characterized as being mild, nonserious, and transient. None of the enzyme elevations were accompanied by cholestatic markers, nor were the criteria for Hy's law (hepatocellular damage with jaundice but no cholestasis, a risk factor for hepatic failure) met by any participant. Elevations in the alanine aminotransferase level were managed with glucocorticoids as described in the Methods section.

The first participant in cohort 3 (Participant 3) had an elevation in the alanine aminotransferase level, so prophylactic glucocorticoids were administered in the remaining participants in that cohort. In cohort 4, glucocorticoids were used in an "on-demand" manner, only in response to elevations of the alanine aminotransferase level in four of the six participants.

Glucocorticoids were not administered to Participants 1, 2, 10, and 13. Glucocorticoids were initiated at a dose of 60 mg per day after the alanine aminotransferase level was elevated to 1.5 times the baseline value in Participants 3, 6, 9, 11, and 12; glucocorticoids were also initiated after the alanine aminotransferase level was elevated to 1.5 times the upper limit of the normal range in Participants 14 and 15. Participants 4, 5, 7, and 8 received prophylactic glucocorticoids with oral prednisone, initiated at a dose of 40 mg per day, 3 weeks after the infusion, for a period of 4 weeks; glucocorticoids were slowly tapered, for a total of at least 15 weeks of therapy.

As specified in the protocol, investigators could adjust glucocorticoid treatment schedules on the basis of alanine aminotransferase levels. In general, the use of prophylactic glucocorticoids was associated with a delayed time until the first elevation of the alanine aminotransferase level and a shorter duration of having the alanine aminotransferase level above the upper limit of the normal range than was observed with on-demand glucocorticoid use.

Consistent with previously reported findings,[1] no apparent association was noted between elevation of the alanine aminotransferase level and anticapsid T-cell response (see the Immune Response Monitoring section in the Supplementary Appendix).[10,11] No participant reported symptoms

Figure 1 (facing page). Individual Profiles for Participants 6, 7, and 8.

Factor VIII activity levels (blue bars), factor VIII use (see color key for reasons), alanine aminotransferase (ALT) levels (yellow lines), and glucocorticoid use (green bar below each graph) are shown over time for Participants 6, 7, and 8. These participants were in cohort 3, in which they received 6×10^{13} vector genomes (vg) per kilogram of body weight. Factor VIII activity was assessed by means of chromogenic assay. The dashed line indicates the upper limit of the normal range (ULN) for ALT. Profiles for all study participants are shown in Figure S1 in the Supplementary Appendix.

that were suggestive of liver dysfunction in conjunction with an elevated alanine aminotransferase level or that showed any long-term effect on liver function. The γ-glutamyltransferase enzyme levels were monitored as a sensitive indicator of hepatotoxicity, and no safety signal emerged.

Three participants reported serious adverse events during the study. Participant 13 was hospitalized for observation after grade 2 pyrexia developed along with myalgia and headache within 24 hours after the administration of AAV5-hFVIII-SQ. This serious adverse event was characterized by the investigator as being related to treatment on the basis of temporal proximity to the infusion, and the symptoms resolved within 48 hours after the participant received acetaminophen. Participants 3 and 6 had serious adverse events associated with preexisting hemophilic arthropathies that led to hospitalizations for elective total knee-replacement surgeries; both events were characterized by investigators as not being related to treatment.

LONG-TERM CLINICAL EFFECTIVENESS OF AAV5-hFVIII-SQ

Three-Year Follow-up in Cohorts 1 and 2

The one participant in cohort 1 (who had received 6×10^{12} vg per kilogram) and the one participant in cohort 2 (who had received 2×10^{13} vg per kilogram) continued to have factor VIII expression levels below 1 IU per deciliter, as assessed on chromogenic assay. Monitoring is ongoing for both participants.
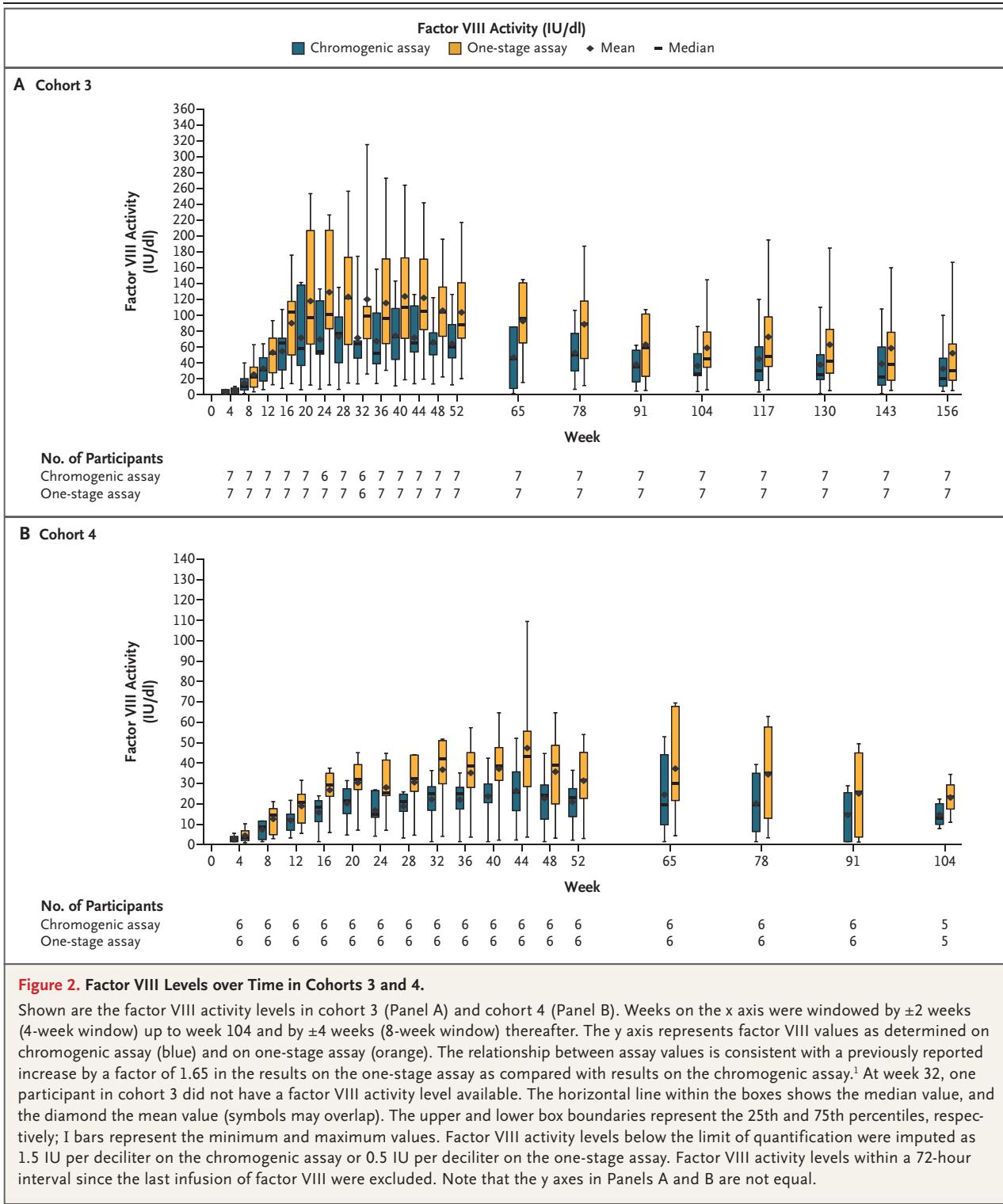
Three-Year Follow-up in Cohort 3

The seven participants in cohort 3, who had received 6×10^{13} vg per kilogram, had the following mean factor VIII activity levels as measured by chromogenic assay at the end of years 1, 2, and 3:

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Figure 2. Factor VIII Levels over Time in Cohorts 3 and 4.

Shown are the factor VIII activity levels in cohort 3 (Panel A) and cohort 4 (Panel B). Weeks on the x axis were windowed by ±2 weeks (4-week window) up to week 104 and by ±4 weeks (8-week window) thereafter. The y axis represents factor VIII values as determined on chromogenic assay (blue) and on one-stage assay (orange). The relationship between assay values is consistent with a previously reported increase by a factor of 1.65 in the results on the one-stage assay as compared with results on the chromogenic assay.[1] At week 32, one participant in cohort 3 did not have a factor VIII activity level available. The horizontal line within the boxes shows the median value, and the diamond the mean value (symbols may overlap). The upper and lower box boundaries represent the 25th and 75th percentiles, respectively; I bars represent the minimum and maximum values. Factor VIII activity levels below the limit of quantification were imputed as 1.5 IU per deciliter on the chromogenic assay or 0.5 IU per deciliter on the one-stage assay. Factor VIII activity levels within a 72-hour interval since the last infusion of factor VIII were excluded. Note that the y axes in Panels A and B are not equal.

64 IU per deciliter (median, 60 IU per deciliter), 36 IU per deciliter (median, 26 IU per deciliter), and 33 IU per deciliter (median, 20 IU per deciliter), respectively (Figs. 1 and 2 and Fig. S2). Factor VIII expression changed as a function of time and the peak factor VIII level reached; the mean factor VIII expression decreased by 43% over the course of year 2 and by 10% over the course of year 3. The results on the one-stage assay consistently showed factor VIII levels that were approximately 1.6 times as high as those observed on chromogenic assay, as reported previously.[1] At the ends of years 1, 2, and 3, the mean factor VIII levels according to the one-stage assay were as follows: 104 IU per deciliter (median, 89 IU per deciliter), 59 IU per deciliter (median, 46 IU per deciliter), and 52 IU per deciliter (median, 30 IU per deciliter), respectively, with a nonhemophilic value defined as a level greater than 40 IU per deciliter.

The mean annualized rate of bleeding events decreased by 96%, from a mean (±SD) of 16.3±15.7 events per year (median, 16.5 events per year) at baseline to 0.7±1.6 events per year (median, 0.0 events per year) at the end of year 3 (Fig. 3). In the year before study entry, only one participant who was receiving prophylaxis was free from participant-reported breakthrough bleeding events that led to additional factor VIII treatment. At the end of study year 3, a total of six participants (86%) were free from bleeding events. All the participants in this cohort had full resolution of bleeding in target joints (or arthropathy)[12] by year 2. (Target joints are major joints with ≥3 bleeding events within 6 months; bleeding in target joints is considered to be resolved if there are ≤2 bleeding events within 12 months.)

In the year before study entry, the mean annualized number of factor VIII infusions per participant was 136.7±22.4 (median, 138.5); at the end of year 3, the mean annualized use of exogenous factor VIII decreased by 96% to a mean of 5.5±9.4 infusions (median, 0.0 infusions) (Fig. 3). A total of 98% of the factor VIII infusions after post-administration week 5 were reported by Participants 3 and 6, in association with two total knee-replacement surgeries that had been necessitated by preexisting hemophilic arthropathies. A total of 11 treated bleeding events were reported after week 5, with 10 of these events occurring in Participant 6, who had the lowest factor VIII expression after the infusion.
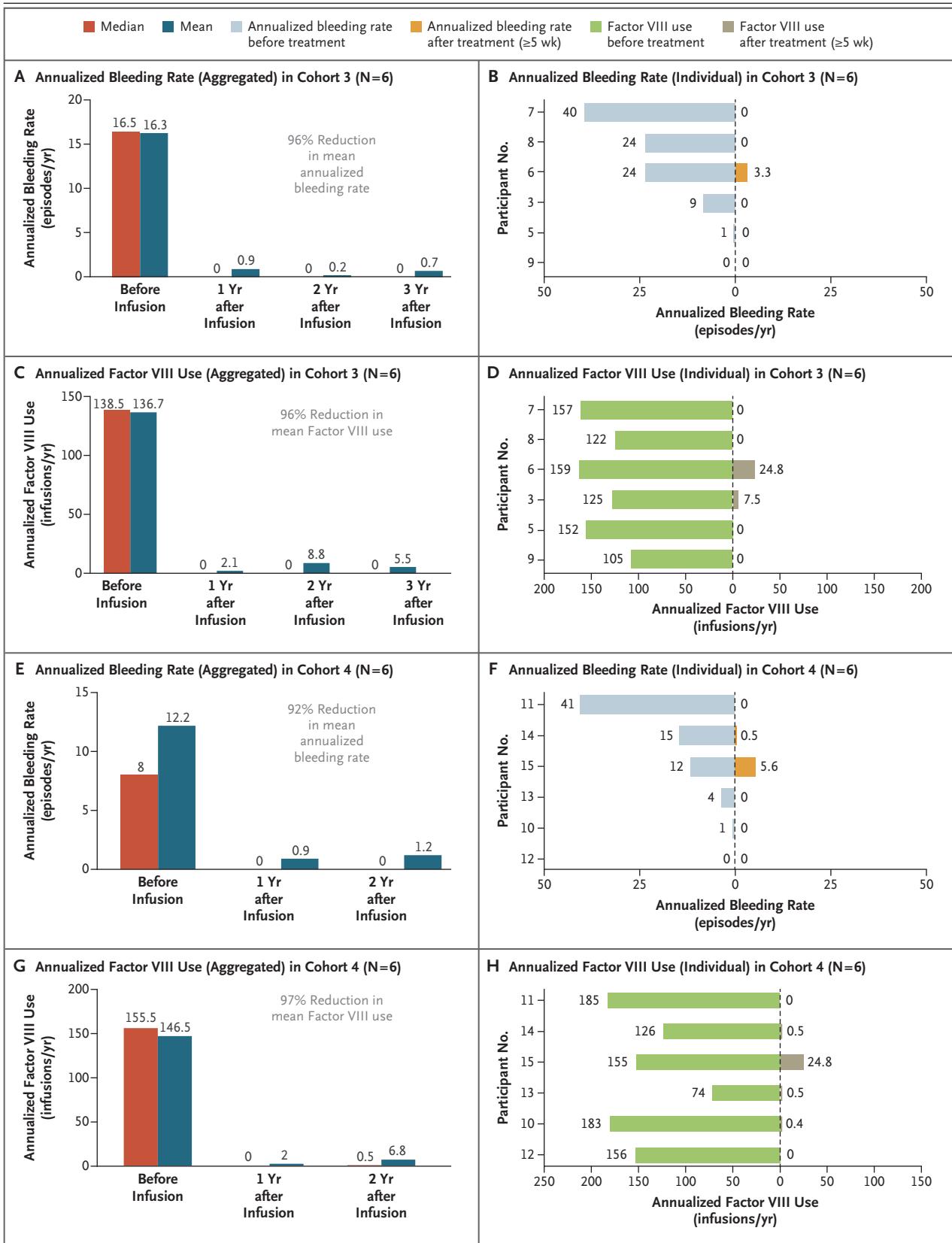
Two-Year Follow-up in Cohort 4

The six participants in cohort 4, who had received 4×10^{13} vg per kilogram, had a mean factor VIII activity level of 21.0 IU per deciliter (median, 23 IU per deciliter) at the end of year 1 and 15 IU per deciliter (median, 13 IU per deciliter) at the end of year 2 (Figs. 1 and 2 and Fig. S1). The level decreased by 30% over the course of year 2 and followed a trajectory similar to that seen in cohort 3. According to the one-stage assay, the mean factor VIII levels were 31 IU per deciliter (median, 32 IU per deciliter) at the end of year 1 and 23 IU per deciliter (median, 24 IU per deciliter) at the end of year 2.

The annualized rate of bleeding events decreased by 92%, from a mean of 12.2±15.4 events per year (median, 8.0 events per year) in the year before study entry to a mean of 1.2±2.4 events per year (median, 0.0 events per year) at the end of year 2 (Fig. 3). In the year before study entry, 17% of the participants were free from bleeding events that led to factor VIII treatment for breakthrough bleeding; 2 years after the infusion, 67% of the participants were free from bleeding events. In the year before study entry, the mean annualized number of factor VIII infusions per participant was 146.5±41.6 (median, 155.5). At end of year 2, the mean annual use of exogenous factor VIII decreased by 95%, to a mean of 6.8±15.6 infusions (median, 0.5 infusions) (Fig. 3).

With the exception of Participant 15, all the participants in cohort 4 had full resolution of bleeding in target joints[12] by year 2. Participant 15 reported 11 of the 12 bleeding events and 94% of the use of factor VIII that occurred in cohort 4 after post-administration week 5.

FACTOR VIII LEVELS AND CLINICAL OUTCOMES

The relationship between factor VIII level and bleeding risk is challenging to describe in severe hemophilia A, since factor VIII levels fluctuate in response to infusions of factor VIII replacement therapy. Data from this study showed an asymptotic reduction in the frequency of bleeding events as factor VIII activity levels increased

Legend: ■ Median ■ Mean ■ Annualized bleeding rate before treatment ■ Annualized bleeding rate after treatment (≥5 wk) ■ Factor VIII use before treatment ■ Factor VIII use after treatment (≥5 wk)

A Annualized Bleeding Rate (Aggregated) in Cohort 3 (N=6)

B Annualized Bleeding Rate (Individual) in Cohort 3 (N=6)

C Annualized Factor VIII Use (Aggregated) in Cohort 3 (N=6)

D Annualized Factor VIII Use (Individual) in Cohort 3 (N=6)

E Annualized Bleeding Rate (Aggregated) in Cohort 4 (N=6)

F Annualized Bleeding Rate (Individual) in Cohort 4 (N=6)

G Annualized Factor VIII Use (Aggregated) in Cohort 4 (N=6)

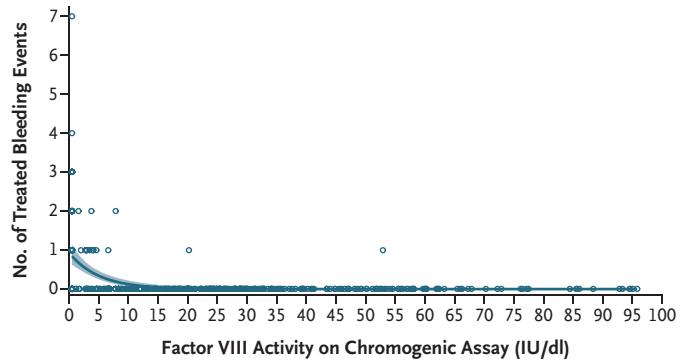**H** Annualized Factor VIII Use (Individual) in Cohort 4 (N=6)

Figure 3 (facing page). Annualized Rates of Bleeding Events and Factor VIII Use.

Shown are the annualized rates of bleeding events among participants in cohort 3 in aggregate (Panel A) and individually (Panel B), as well as the factor VIII utilization data for cohort 3 in aggregate (Panel C) and individually (Panel D). Data in Panels A and B are shown only for the participants who were treated prophylactically in the year before the start of the study. The rate of mean annualized infusions of exogenous factor VIII relative to the mean annualized rate of bleeding events after week 5 was attributable in part to prophylactic factor VIII use during two non–treatment-related surgical events. A total of 10 bleeding events (91% of the overall events) and 75 events of factor VIII use (74% of the overall events) occurred in Participant 6, who underwent total knee-replacement surgery for preexisting, chronic hemophilic arthropathy and who had the lowest post-treatment factor VIII activity in the cohort. Participant 3 had no bleeding events and 24 events of factor VIII use (24% of the overall events), which also reflected prophylactic administration during total knee-replacement surgery for preexisting, chronic hemophilic arthropathy. Also shown are the annualized rates of bleeding events among participants in cohort 4 in aggregate (Panel E) and individually (Panel F), as well as the factor VIII utilization data for cohort 4 in aggregate (Panel G) and individually (Panel H). Of 12 bleeding events and 52 events of factor VIII use that occurred after week 5 in this cohort, 11 bleeding events (92% of the overall events) and 49 events of factor VIII use (94% of the overall events) occurred in Participant 15, who consistently had lower factor VIII levels than the rest of the cohort. Interrogation of this participant's background information (including medical history, concomitant medication use, and laboratory findings) did not reveal any clear reason for factor VIII expression levels being lower than in cohort counterparts (Fig. S1).

Figure 4. Number of Observed and Predicted Bleeding Events, According to Factor VIII Activity Level.

Shown are the numbers of participant-reported treated bleeding events according to factor VIII activity level in all the participants. Open circles represent the number of observed participant-reported treated bleeding events and the corresponding factor VIII level (as assessed on chromogenic assay) for a single participant within a 4-week window. Predicted values (shaded line) are based on negative binomial regression. Two outlying data points are observed in this representation. The first corresponds to a mean factor VIII level of 20 at weeks 4 to 8 in Participant 4; the per-week values during this time were 5 IU per deciliter, 12 IU per deciliter, 152 IU per deciliter, and 49 IU per deciliter. Factor VIII was administered at the point when the levels were approximately 5 IU per deciliter, in response to a muscular bleeding event. The second outlier corresponds to a self-administration of factor VIII by Participant 14 in response to a traumatic ankle injury, at circulating factor VIII levels of 53 IU per deciliter.

INDIVIDUAL PARTICIPANT PROFILES

Decrease and Persistence in Factor VIII Expression

Individual patterns of factor VIII expression commonly showed slow initial increases in circulating factor VIII levels over the first 20 to 28 weeks, followed by peaking and then a gradually slowing decrease (Figs. 1 and 2 and Figs. S1 and S2). Underlying this trend were three detectable stages of DNA microdistribution and structural dynamics, as assessed with the use of whole blood cells as an observable proxy for hepatocytes and with the use of droplet digital polymerase chain reaction to detect the formation of full-length expression cassettes and inverted-terminal-repeat fusion events, respectively (Fig. S3).

Slow initial increases in factor VIII expression are consistent with preclinical studies of AAV5-hFVIII-SQ[8] and other AAV5 vectors[14] and are due in part to rates of viral uncoating and DNA delivery.[15] During the slow increase in factor VIII protein expression, we observed an approximately 4-log decline in non–full-length factor VIII DNA

(Fig. 4). This finding is in alignment with previously published data in populations of patients with hemophilia A of differing severities[13] and provides further evidence of equivalent biologic activity between the AAV5-hFVIII-SQ transgene and native factor VIII.

At end of year 3, the levels of factor VIII that were measured by the more conservative chromogenic assay placed the participants in cohort 3 into categories of hemophilia A that were defined as nonhemophilic (one participant), mild (five participants), and moderate (one participant). At this same time point, all the participants were no longer receiving prophylaxis, had a mean annualized rate of bleeding events of 0, and had full resolution of bleeding in target joints.

coinciding with the life span of short-lived granulocytes, followed by a less-rapid decline associated with the life span of nonnucleated red cells (Fig. S4). Despite bulk loss of vector DNA in this period of increasing protein expression, the portion of transgene DNA in peripheral-blood mononuclear cells showed stronger persistence, presumably owing to an observed simultaneous increase in the percentage of stable circularized DNA episomes in the nuclei of peripheral-blood mononuclear cells (Fig. S4), a finding that confirms a previously established mechanism of vector DNA persistence.[16-18]

Finally, a slowing decrease in factor VIII expression was observed in years 2 and 3, which potentially reflects a gradual turnover of stable, expression-competent DNA in longer-lived, nucleated cells. Thus, persistent transgene expression through years 2 and 3 was consistent with the establishment of stable episomal transgenes detected in observable nucleated cells during year 1. Hepatocytes are presumed to be the predominant host of longer-term AAV5-hFVIII-SQ–derived factor VIII protein expression owing to AAV5 tropism and the use of a liver-specific promoter; liver-biopsy studies are under way.

Host Response Variability

Each biologic step occurring between the administration of gene therapy and transgene expression may vary among hosts.[16,17] Responses to gene therapy can also vary between serotypes and transgenes. This variation is particularly likely for factor VIII, owing to manufacturing and processing complexities caused by its large size and extensive post-translational modifications.[19,20] Indeed, factor VIII expression shows variability within and among healthy adults.[21-26] Mechanisms of gene-therapy response variability due to individual-specific biologic variables remain to be understood, but an initial investigation into participant-specific biologic variables was undertaken within the limitations of the small sample population studied here. Individual participant profiles and medical records were interrogated to provide information about causality of variability in factor VIII expression (Fig. 1 and Fig. S1).

In cohort 3, all the participants had a mean factor VIII level within the ranges considered to indicate mild hemophilia A or nonhemophilic status, except for Participant 6, who had a mean factor VIII level that was within the range considered to indicate moderate hemophilia A, according to the chromogenic assay (4 IU per deciliter), or considered to indicate the mild range, according to the one-stage assay (6 IU per deciliter). Similarly, in cohort 4, all the participants had a mean factor VIII expression level associated with the mild range of hemophilia in year 2. However, Participant 15 (who had a mean factor VIII level of 6 IU per deciliter, according to the chromogenic assay) had the majority of the post-administration bleeding events and factor VIII utilization in the cohort, as well as having the only target joint with bleeding that did not resolve in the study. Individual response profiles and medical histories for Participants 6 and 15 were compared with those of the cohort counterparts (Fig. S1). Participant 6 had an early elevation in the alanine aminotransferase level that was treated with on-demand glucocorticoids before the planned initiation of prophylactic glucocorticoids; however, Participant 9 had a similar experience, including initiation of on-demand glucocorticoids before the planned initiation of prophylactic glucocorticoids, and had a mean factor VIII expression level that was nine times as high as that in Participant 6, with a similar clinical benefit being observed in both participants. Participant 15 had no potentially causal differences from other participants in various factors such as the time until the initiation of glucocorticoids after the elevation in the alanine aminotransferase level, glucocorticoid dosage, or glucocorticoid tapering. An examination of all the response profiles broadly suggests that glucocorticoids may have assisted in rescue or protection of factor VIII levels during elevations of the alanine aminotransferase level (in Participants 3, 5, 8, 9, 11, 12, and 14) and in resolution of elevated alanine aminotransferase levels (in Participants 3, 4, 5, 12, 14, and 15). The elevations in the alanine aminotransferase level appeared to have been temporally associated with decreases in factor VIII expression in some participants (Participants 4, 5, 7, and 8).

Additional variables under assessment included: the timing, dose, duration, and tapering of the glucocorticoid regimen; the historical annualized rate of bleeding events, pharmacokinetics, and type of previous use of exogenous factor VIII; concomitant medications; ABO blood type; von Willebrand factor level; HLA type; age; body-

mass index and weight; liver health as assessed by means of C-reactive protein level, hepatitis status, liver-function tests, and FibroTest; vector infusion variables, including the type of pump, duration, and presence of extravasation; and adverse events.

Owing to the small sample size, no specific variables contributing to individual factor VIII levels could be identified in this study. However, trends in the timing of glucocorticoid administration relative to elevations in the alanine aminotransferase level are suggestive.

DISCUSSION

This study of AAV5-hFVIII-SQ showed that an oversized gene could be packaged into an AAV capsid, achieve full length and inverted-terminal-repeat fusion within cell nuclei, and express functional transgene to yield sustained clinical benefit. These data show multiyear factor VIII expression and effective control of bleeding in 13 men with severe hemophilia A. Within the most recent year of follow-up, participants had factor VIII levels within the nonhemophilic range (1 participant), the mild hemophilic range (11 participants), and the moderate hemophilic range (1 participant), as measured by the chromogenic assay (which is more conservative than the one-stage assay).

No new safety events were noted.[1] All elevations in the alanine aminotransferase level were mild, nonserious, and transient, and there were no symptoms or sequelae suggestive of clinically significant hepatocyte injury or liver dysfunction. No cellular immune responses to factor VIII or AAV5 capsid were consistently detected in any participant. No development of factor VIII inhibitors or other factor VIII antibodies was detected.

Liver health was evaluated to assess the burden on hepatocytes to produce B-domain–deleted factor VIII and eliminate viral capsids after transgene DNA delivery or fragmented vector DNA. The levels of alanine aminotransferase and γ-glutamyltransferase did not indicate ongoing liver damage, and, in the absence of detectable cellular immune response, decreases in factor VIII expression and elevations in the alanine aminotransferase level cannot be attributed to abnormal hepatocyte turnover. No evidence of endoplasmic reticulum stress was noted at doses explored in this study, but studies in mice and nonhuman primates have shown that AAV-mediated constructs containing factor VIII and a stronger promoter than that of AAV5-hFVIII-SQ can cause endoplasmic reticulum stress at higher doses than those studied here.[8,27] Liver-biopsy studies involving humans will be necessary to confirm the translatability of these results.

Participants in cohorts 3 and 4 had a slow increase to varying peak levels of factor VIII expression, followed by decreasing rates of decline over time, with exceptions. Variability in factor VIII levels has been observed in healthy persons, which suggests that normal biologic variables could contribute to observed differences in factor VIII expression through the activities of trans-acting genetic modifiers, age, von Willebrand factor level, lipoprotein receptor–related protein activity, and metabolism.[21-26] An initial investigation into potential mechanisms underlying response variability yielded no significant signals, but observed trends suggest that glucocorticoid regimens may play a role. Findings from this study and others underscore the importance of achieving a high initial transgene expression.

Additional mechanistic hypotheses of variability deserve further exploration and relate to the complexity and potential stress of factor VIII assembly and folding, host cell type, potential destabilization or silencing of nuclear factor VIII DNA, potential threshold response,[28] and hepatocyte AAV receptor polymorphisms and distribution. Finally, lifestyle factors such as liver health, trauma, metabolism, alcohol consumption, and acetaminophen use may introduce variability.

Persistent factor VIII expression is associated with the formation of stable, circularized DNA in cell nuclei. Experiments in mice have shown that factor VIII levels in plasma are representative of vector DNA levels in hepatocytes,[8] but there are limitations to studying nucleated blood cells as a proxy for hepatocyte transduction, owing in part to potential differences in cell-type–specific transduction mechanisms. To better understand DNA structural dynamics that may underlie persistent expression, transduction in hepatocytes will need to be specifically studied by means of liver biopsy.

These long-term follow-up data contribute to a growing understanding of AAV vector biology and to our progress toward a useful treatment for persons living with hemophilia A. At the latest time point assessed, the 13 participants in co-

horts 3 and 4 in our study had substantial reductions in the incidence of bleeding events, had resolution of bleeding in target joints (in 12 participants), and had complete cessation of factor VIII prophylaxis. Monitoring of these participants is continuing; efficacy and safety are being examined in an ongoing expanded phase 3 study (ClinicalTrials.gov number, NCT03370913; EudraCT number, 2017-003215-19).

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