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Activity of Transgene-Produced B-Domain Deleted Factor VIII in Human Plasma Following AAV5 Gene Therapy

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Abstract:
Adeno-associated virus (AAV)-based gene therapies can restore endogenous factor VIII (FVIII) expression in hemophilia A (HA). AAV vectors typically utilize a B-domain deleted FVIII transgene, such as human FVIII-SQ in valoctocogene roxaparvovec (AAV5-FVIII-SQ). Surprisingly, the activity of transgene-produced FVIII-SQ was between 1.3 and 2.0 times higher in one-stage clot (OS) than chromogenic-substrate (CS) assays, while recombinant FVIII-SQ products have lower OS than CS activity. Transgene-produced and recombinant FVIII-SQ showed comparable specific activity (IU/mg) in the CS assay, demonstrating that the diverging activities arise in the OS assay. Higher OS activity for transgene-produced FVIII-SQ was observed across various assays kits and clinical laboratories, suggesting intrinsic molecular features as a potential root cause. Further experiments in two participants showed that transgene-produced FVIII-SQ accelerated early factor Xa and thrombin formation, which may explain the higher OS activity based on a kinetic bias between OS and CS assay readout times. Despite the faster onset of coagulation, global thrombin levels were unaffected. A correlation with joint bleeds suggested that both OS and CS assay remained clinically meaningful to distinguish hemophilic from non-hemophilic FVIII activity levels. During clinical development, the CS activity was chosen as a surrogate endpoint to conservatively assess hemostatic efficacy and enable comparison with recombinant FVIII-SQ products.

Relevant trials are registered on Clinicaltrials.gov (NCT02576795 and NCT03370913).

Conflict of interest: COI declared - see note

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ABSTRACT

Adeno-associated virus (AAV)-based gene therapies can restore endogenous factor VIII (FVIII) expression in hemophilia A (HA). AAV vectors typically utilize a B-domain deleted FVIII transgene, such as human FVIII-SQ in valoctocogene roxaparvovec (AAV5-FVIII-SQ). Surprisingly, the activity of transgene-produced FVIII-SQ was between 1.3 and 2.0 times higher in one-stage clot (OS) than chromogenic-substrate (CS) assays, while recombinant FVIII-SQ products have lower OS than CS activity. Transgene-produced and recombinant FVIII-SQ showed comparable specific activity (IU/mg) in the CS assay, demonstrating that the diverging activities arise in the OS assay. Higher OS activity for transgene-produced FVIII-SQ was observed across various assays kits and clinical laboratories, suggesting intrinsic molecular features as a potential root cause. Further experiments in two participants showed that transgene-produced FVIII-SQ accelerated early factor Xa and thrombin formation, which may explain the higher OS activity based on a kinetic bias between OS and CS assay readout times. Despite the faster onset of coagulation, global thrombin levels were unaffected. A correlation with joint bleeds suggested that both OS and CS assay remained clinically meaningful to distinguish hemophilic from non-hemophilic FVIII activity levels. During clinical development, the CS activity was chosen as a surrogate endpoint to conservatively assess hemostatic efficacy and enable comparison with recombinant FVIII-SQ products.

Relevant trials are registered on Clinicaltrials.gov (NCT02576795 and NCT03370913).

KEY POINTS

- Higher OS than CS activity for AAV5-FVIII-SQ is likely caused by accelerated early FXa formation, resulting in a kinetic bias between assays.
- Specific activity (IU/mg) remains comparable between transgene-produced and recombinant FVIII-SQ in the CS assay, but not in the OS assay.

INTRODUCTION

Measurement of coagulation factor activity has emerged as a key endpoint for determining treatment success of adeno-associated virus (AAV)-based gene therapies for inherited bleeding disorders, such as hemophilia A (HA). HA is caused by genetic deficiency in coagulation factor VIII (FVIII), and AAV gene therapies aim to complement this deficiency by providing a shortened, yet fully functional version of FVIII; for example, B-domain-deleted (BDD) FVIII-SQ in valoctocogene roxaparvovec (AAV5-FVIII-SQ, BMN 270) (Sup. Fig. 1).[1] Given the monogenic nature of HA, increased FVIII activity in plasma should ameliorate the bleeding phenotype and be a predictive marker for clinical outcomes. This has implications for patient management and drug development, since using FVIII activity as an endpoint allows for a more objective efficacy evaluation than using participant-reported annualized bleed rates.

FVIII activity is measured with 2 different assays: one-stage clot (OS) and chromogenic-substrate (CS) assays. However, it became clear shortly after initiation of the first AAV gene therapy trial in HA (trial 270-201; NCT02576795) that these 2 assays did not yield identical results.[1] HA participants treated with a single dose of BMN 270 gene therapy showed a plasma FVIII activity that was on average approximately 1.65 times higher in the OS compared to the CS assay. In contrast, recombinant BDD-FVIII products typically show lower OS than CS activity.[2-6] Higher OS than CS activity was previously reported for certain purified FVIII standards, such as the Mega 2 Concentrate, possibly caused by FVIII modifications during lyophilization that promote faster activation, resulting in 1.3 times higher OS values.[7,8] Higher OS activity was also observed in mild HA, associated with particular FVIII missense mutations;[9-11] in one report, OS results were 1.67 to 4 times higher than those from the CS assay.[12] Yet, since AAV gene therapy-derived FVIII-SQ is not processed in vitro and contains a wildtype consensus amino acid sequence, these prior instances did not offer an immediate explanation for the discrepant activities.

With the anticipated clinical use of AAV gene therapies in HA, we sought to better understand how the activity of transgene-produced FVIII-SQ can be reliably determined. In a field already notorious for assay discrepancies, it was also critical to compare transgene-derived FVIII activity to the activity of recombinant FVIII products, which have served as the standard of care over the

past 3 decades. To these ends, we used several approaches, including a clinical laboratory field study encompassing different OS and CS assay kits, determination of specific FVIII activity (IU/mg), production of FVIII-SQ in different cell types, kinetic characterization of the coagulation pathway, and a correlation analysis between joint bleeds and FVIII activity.

MATERIALS AND METHODS

Plasma Samples

Plasma from individuals treated with BMN 270 in trial 270-201 (Clinicaltrials.gov NCT02576795; EudraCT 2014-003880-38; n=13) and 270-301 (NCT03370913; 2017-003215-19; n=22), sponsored by BioMarin, was procured in accordance with Clinical & Laboratory Standards Institute (CLSI) guideline H21-A5, Vol 28, No 5. Plasma from healthy donors and pooled plasma quality controls (QCs) (CRYOcheck™ PNP, ARP1, ARP2) were from Precision, diluted into FVIII buffer (Chromogenix) as needed. Pooled congenital FVIII-deficient plasma (HRF or George King) was used for ReFacto®-spiked QCs. The study protocols were approved by local Institutional Review Boards or Institutional Ethics Committees, and patients gave informed consent.

OS FVIII Activity Assay

Samples were mixed 1:1 with immuno-depleted FVIII-deficient plasma (Siemens), APTT reagent (Actin FSL; Siemens), and $CaCl_2$ (Siemens) on BCS® XP analyzer (Siemens), and time to clot formation was measured optically. A 7-point high (1.500 to 0.050 IU/mL) and a 7-point low (0.150 to 0.0025 IU/mL) calibration curve were prepared using CRYOcheck™ NRP (Precision). The high curve prepared in Tris/BSA buffer (50 mM Tris, 150 mM NaCl, and 1% BSA) was tested 1:2 diluted. The low curve prepared in FVIII-deficient plasma was tested neat. Calibration was performed in duplicate, using linear log-lin regression. Clinical samples were tested in duplicate at 3 dilutions (1:2, 1:4 and 1:8 in Tris/BSA for high curve; neat, 1:2 and 1:4 in FVIII-deficient plasma for low curve). A minimum of 2 dilutions within ±15% of the mean were required, except for samples ≤ 0.050 IU/mL, for which the least dilute result was reported.

CS FVIII Activity Assay

Samples were mixed with phospholipids, FIXa, excess FX, and $CaCl_2$ on BCS®XP, followed by addition of FXa substrate S-2765 (Z-D-Arg-Gly-Arg-pNA), using the Coatest® SP4 FVIII kit (Chromogenix), and the rate of color formation was measured optically at 405 nm (kinetic

mode). An 8-point high (1.500 to 0.150 IU/mL) and an 8-point low (0.200 to 0.0075 IU/mL) calibration curve were prepared using CRYOcheck™ NRP (Precision). The high curve, prepared in Coatest® FVIII buffer (1% BSA), was tested 1:12 diluted. The low curve, prepared in congenital FVIII-deficient plasma (Helena), was tested 1:5 diluted. Calibration was performed in duplicate, using linear lin-lin regression. Clinical samples were tested in duplicate at 3 dilutions (1:12, 1:24 and 1:48 in Coatest® FVIII buffer for high curve; 1:5, 1:10, and 1:20 in congenital FVIII-deficient plasma for low curve). A minimum of 2 dilutions within ±15% of the mean were required, except for samples ≤ 0.060 IU/mL, for which the least dilute result was reported.

BDD FVIII-SQ ELISA

After coating with antibody (GMA-8024, Green Mountain) and blocking, samples were diluted 1:50 in Sample Diluent and added to the plate. After incubation, biotinylated detection antibody (GMA-8023, Green Mountain) was added, followed by streptavidin-horseradish peroxidase, 3,3', 5,5'-Tetramethylbenzidine substrate and acidic stop solution. Absorbance was measured at 450 nm. A 10-point standard curve with 4 anchor points (range of quantification: 4.7 ng/mL to 80.8 ng/mL) was prepared using ReFacto® AF/Xyntha® (Pfizer/Wyeth) in FVIII-deficient plasma (George King), diluted in Sample Diluent, and regressed using a 4-parameter logistic fit. Samples were tested in duplicate; samples above 80.8 ng/mL were diluted and retested.

Thrombin Generation Assay (TGA)

Fluorogenic thrombin substrate, MP reagent (Stago), and 1000 pM recombinant FIXa (Haematologic Technologies) were added to samples. Cleaved substrate was monitored at 37°C on Fluoroskan Ascent® (Thermo), correcting for α2M-thrombin and inner-filter effect by continuous individual calibration. Data were collected using Thrombinoscope® software (Stago).

FIXa Kinetic Studies

Normal plasma comparators consisted of CRYOcheck™ NRP (Precision) in FVIII-deficient plasma (Siemens). Samples were diluted (50 mM imidazole [pH 7.3], 100 mM NaCl, 1% [w/v]

BSA), mixed with FVIII-deficient plasma, and incubated for 7-8 min at 37°C (Micro-Hywel), followed by APTT addition (Actin FSL; Siemens). After 2.5 min, a t_0 sub-sample was collected. After 3 min, $CaCl_2$ was added and subsamples were collected immediately and approximately every 8-9 sec into an equal volume of ice-cold FIXa Stop Solution.[13] An aliquot was frozen at -70°C for FXa and FIIa quantification. The remaining subsample was used for FIXa quantification (ROX FIX-A kit, Rossix): Briefly, subsamples were diluted in ice-cold buffer and placed into a microplate with ice-cold calibrators. The plate was heated at 37°C for 3 min, followed by addition of Reagent 1 and Reagent 2. After 4 min at 37°C, FXa substrate (S-2765) was added for 1 to 3 min, followed by 2% citric acid Stop Solution. Absorbance was measured at 405 nm on ThermoMax (Molecular Devices).

FXa and FIIa Kinetic Studies

Kinetic experiments were performed as above. FXa in stopped subsamples was quantified using a prothrombinase-complex method that measures FXa-mediated FIIa (thrombin) formation. Briefly, subsamples or human FXa calibrators (Enzyme Research) were incubated at 37°C for 3 min, followed by addition of human FII (Rossix), bovine FVa (Rossix), Phospholipid-TGT (Rossix), and $CaCl_2$. During a 3-min activation at 37°C, prothrombinase (FXa+FVa) catalyzed the conversion of FII (prothrombin) into FIIa (thrombin), quantified by using a FIIa substrate S-2238 (H-D-Phe-Pip-Arg-pNA, Chromogenix). Substrate hydrolysis was stopped with 2% citric acid after 6 min at 37°C. Absorbance was measured at 405 nm on ThermoMax. Thrombin in the original subsamples was also quantified using direct hydrolysis of S-2238 and stopped with 2% citric acid after 2 h at 37°C.

Statistical Analysis of OS and CS FVIII Activity

Linear regressions for post-baseline, visit-matched OS/CS FVIII activity were generated using SAS (version 9.4). Data were excluded if obtained within 72 hours or 3 calendar days of the last FVIII infusion, or if below the limit of quantification (BLQ). Statistical outliers (studentized residual > 2 and DFBETAS statistics for slope parameter > 2) were removed. For healthy donors,

the y intercept was suppressed; without suppression, the y intercept was 9.167, the slope was 0.962, and R^2 was 0.752.

Statistical Analysis of Specific FVIII Activity

Post-baseline FVIII activity was normalized to visit-matched FVIII-SQ protein. Intra- and inter-participant mean, standard deviation or range were calculated. Data were excluded if obtained within 72 hours or 3 calendar days of the last FVIII infusion; BLQ activity was imputed as 0 IU/dL. Participants who did not have at least one quantifiable specific activity result for both OS and CS assay were excluded.

Statistical Analysis of Joint Bleed Frequency and FVIII Activity Measurements

Negative binomial regression was applied to post-baseline, participant-reported, treated joint bleeds and median FVIII activity in 4-week intervals using SAS. Joint bleed frequency and 95% confidence limits for corresponding FVIII levels were predicted. A generalized estimating equation accounted for correlated responses within the same subject. Data were excluded if obtained within 72 hours or 3 calendar days of the last FVIII infusion; BLQ activity was imputed as 0 IU/dL.

RESULTS

Transgene-Produced FVIII-SQ Has Higher OS Than CS Activity

To compare OS and CS activity for BMN 270 transgene-produced FVIII-SQ in the first-in-human gene-therapy trial 270-201, we extended our previous 1-year analysis [1] by using cumulative 3-year data for cohort 3 (dose: 6 E13 vg/kg) and 2-year data for cohort 4 (dose: 4 E13 vg/kg).[14] The constant rate of change between OS and CS activity was 1.656 (R^2 = 0.963), as evaluated by the slope of the OS/CS regression line (Fig. 1A). Similar results were obtained by linear regression of interim FVIII activity data from the pivotal trial 270-301 (dose: 6 E13 vg/kg), where the OS/CS slope was 1.534 (R^2 = 0.958) (Fig. 1B). In contrast, a linear regression of FVIII activity in 20 healthy donors showed an OS/CS slope of 1.068 (R^2 = 0.99) (Fig. 1C). Furthermore, pooled normal plasma (Sup. Fig 2) and the 6th WHO international standard (Sup Tab. 1) were quantified with comparable FVIII activity in OS and CS assay. Therefore, the consistently higher OS than CS activity measurements were specific to transgene-produced FVIII-SQ.

No Discernible Impact of Assay Reagents or Kits on the OS/CS Difference

Various reagents were investigated in both assays (Sup. Fig. 3-5); none accounted for the higher OS activity of transgene-produced FVIII-SQ. Recombinant BDD-FVIII (Xyntha®/ReFacto® and Novoeight®) showed lower quantification in the OS than CS assay (Sup. Fig. 6), as expected.[2,6,15] To further investigate the potential impact of various OS and CS assay kits, while also accounting for test-site-specific differences, a retrospective field study was conducted across a total of 13 different study sites in trials 270-201 (Sup. Tab. 2) and 270-301 (Sup. Tab. 3). The OS kits at the local laboratories utilized 7 different APTT reagents containing synthetic, animal-derived, or plant-based phospholipids and 3 different types of surface activators (ellagic acid, silica, or kaolin). 6 different CS kits were employed, which contained human or bovine FIXa/FX reagents and utilized 4 different chromogenic FXa substrates. Using linear regression of FVIII activity, we determined the OS/CS slopes for gene-therapy samples in each laboratory, which ranged from 1.292-2.013 across both trials, with over 1,000 unique samples analyzed (Sup. Tab.

2, 3). Thus, higher OS than CS activity was measured for transgene-produced FVIII-SQ, irrespective of assay reagents, kits, or test site. The consistent magnitude and directionality of the OS/CS difference across laboratories suggested that the underlying mechanism is intrinsic to transgene-produced FVIII-SQ and not an artifact of any particular assay kit or testing facility.

Specific Activity for Transgene-Produced and Recombinant FVIII-SQ Are Comparable in the CS Assay

To determine specific FVIII activity, gene-therapy samples with known OS and CS activity were tested in a validated FVIII-SQ protein ELISA. We first confirmed that HA plasma spiked with Xyntha® showed the expected specific activity for recombinant FVIII-SQ products[6,16] using OS and CS assay (Fig. 2A, 2B). For individual gene-therapy participants, the specific activity for transgene-produced FVIII-SQ using the OS assay ranged from 18,331 to 24,650 IU/mg in trial 270-201 (Fig. 2C) and from 13,243 to 34,575 IU/mg in trial 270-301 (Fig. 2E), with an inter-participant average in each trial of 20,481 IU/mg and 21,839 IU/mg, respectively. These values are more than 2 times above the upper limit of specific activity for recombinant FVIII-SQ products using the OS assay.[16] In contrast, the specific activity for transgene-produced FVIII-SQ using the CS assay ranged from 10,581 to 14,031 IU/mg for individual gene-therapy participants in trial 270-201 (Fig. 2D) and from 6,981 to 19,375 IU/mg in trial 270-301 (Fig. 2F), with an inter-participant average in each trial of 12,224 IU/mg and 13,217 IU/mg, respectively. These values fall within the range of specific activity for recombinant FVIII-SQ products using the CS assay.[6] Of note, the apparently higher variability of specific FVIII activity across participants in trial 270-301 (in both assays) was likely confounded by the higher number of participants summarized for this study.

Influence of Sequence Polymorphism, Production Cell Type, and Codon Optimization on the OS/CS Assay Difference

Due to a natural polymorphism, the Xyntha®/ReFacto® sequence differs by one amino acid from the consensus sequence encoded by BMN 270.[17,18] To determine whether this polymorphism (Phe1880Leu) influences the behavior of FVIII-SQ in the assays, we expressed FVIII-SQ^{Leu1880}

(ReFacto-like) or FVIII-SQ^{Phe1880} (BMN270-like) in Chinese Hamster Ovary (CHO) cells. Similar OS/CS or lower OS than CS activity was measured for either sequence variant (Tab. 1, top rows). Thus, the single amino acid exchange is unlikely to account for the lower OS than CS activity of recombinant FVIII-SQ products. Moreover, the decreased OS activity of BMN 270-like FVIII-SQ^{Phe1880} from CHO cells suggested an impact by production cells.

Xyntha®/ReFacto® are manufactured using CHO cells,[19] while gene-therapy FVIII-SQ is produced by hepatocytes within the human body. To directly investigate whether the species of the production cells influences the behavior of FVIII-SQ in the assays, we tested FVIII-SQ purified from transduced human liver carcinoma cells (HepG2), which maintained higher OS than CS activity similar to transgene-produced FVIII-SQ in patient plasma, while CHO-derived Xyntha® showed lower OS measurements (Tab. 1, center rows). Thus, species-specific mechanisms in production cells could influence the diverging behavior of recombinant and transgene-produced FVIII-SQ in the OS assay.

BMN 270 was codon-optimized to facilitate efficient translation in human cells, which may impact FVIII-SQ folding, with potential consequences for stability, protease sensitivity, and function.[20,21] To test whether codon optimization plays a role for the OS/CS difference, FVIII-SQ was expressed in HepG2 cells from either a codon-optimized or non-codon-optimized vector. Higher OS than CS activity was measured for FVIII-SQ from either vector (Tab. 1, bottom rows). To investigate potential tissue-specific differences, human embryonic kidney (HEK) 293 cells were transfected with the codon-optimized vector, which also resulted in higher OS than CS activity for FVIII-SQ. In summary, the higher OS than CS activity of FVIII-SQ expressed in human cells was not a result of codon optimization or liver-specific modifications.

Kinetic Studies Reveal an Accelerated Rate of Early FXa and Thrombin Generation

We reported that transgene-produced FVIII-SQ and native FVIII showed similar FXa kinetics in the CS assay.[1] To investigate the reaction kinetics in the OS assay, samples from two gene-therapy participants were compared to normal plasma in stop kinetics experiments. The comparison was based on the same CS activity to identify any potential kinetic bias between the OS and CS assay. Samples were incubated with APTT reagent; subsamples were collected after addition of $CaCl_2$ and quantified for FIXa, FXa, and FIIa (thrombin). While kinetic profiles for

FIXa generation were indistinguishable between gene-therapy samples and their normal plasma comparators (Fig. 3A, 3B), the quantities of FXa (Fig. 3C, 3D) and thrombin (Fig. 3E, 3F) generated between 40-60 seconds after addition of $CaCl_2$ were elevated for gene-therapy samples, suggesting a mechanism for faster clot formation. Hence, FXa levels generated by transgene-produced FVIII-SQ within the first minute in the OS assay were higher than those expected from its assigned CS activity. Since CS activity is assigned based on FXa levels generated after 5 minutes, these observations suggest that transgene-produced FVIII-SQ selectively accelerates early vs late FXa generation, in comparison to native FVIII. The kinetic bias resulting from the different readout times in OS and CS assays may explain why gene therapy samples have higher FVIII activity in the shorter OS assay.

To confirm that the accelerated onset of coagulation did not result in global overactivation, we used a thrombin generation assay (TGA). Samples collected from gene-therapy participants after they had reached non-hemophilic FVIII activity levels (CS ≥ 40 IU/dL) showed similar endogenous thrombin potential (ETP) and peak height compared to normal plasma, consistent with overall normal coagulation capacity (Fig. 4). Median values for thrombin lag time and time-to-peak were decreased but remained within the normal range, thus confirming a minor acceleration of thrombin formation.

Correlation Between Joint Bleed Frequency and FVIII Activity Measurements

To determine whether OS and CS assay remained relevant for clinical outcomes, a correlation analysis was performed between participant-reported, treated joint bleeds and median FVIII activity in 4-week intervals, using data from gene therapy trials 270-201 (Fig. 5A) and 270-301 (Fig. 5B). Most joint bleeds (42 of 43) reported by 35 participants across both trials occurred at a median FVIII activity of less than 15 IU/dL (OS) and less than or equal to 10 IU/dL (CS). Using a negative binomial model, both OS and CS activity strongly correlated with predicted joint bleed frequency in both trials (P-values ≤ 0.001) (Fig. 5A, B). The regression curve for OS activity was, however, right-shifted compared to that for CS activity, in particular for activity levels < 15 IU/dL. This implies a trend towards higher predicted bleed risk for the same numerical FVIII-SQ activity value, if measured with OS instead of CS assay. Additional clinical data are needed to substantiate this trend, given that the 95% confidence intervals (CI)

overlapped. At and above a median FVIII activity of 40 IU/dL, per definition the lowest non-hemophilic level,[22] the predicted joint bleed frequency was close to zero using either assay (< 0.001 per 4-weeks in CS, < 0.002 per 4-weeks in OS). Therefore, both OS and CS assay remain clinically meaningful to distinguish hemophilic from non-hemophilic conditions after administration of BMN 270 gene therapy.

DISCUSSION

We studied the activity of transgene-produced FVIII-SQ following administration of BMN 270 gene therapy and showed that the higher OS than CS activity was likely caused by an accelerated onset of FXa and thrombin formation (Fig. 3). This would predominantly impact the OS assay due to a shorter readout time (Fig. 6). TGA assessments provided no evidence for increased thrombotic risk, since neither ETP nor peak height were elevated (Fig. 4).[23-25] Further, the specific activity (IU/mg) of transgene-produced and recombinant FVIII-SQ (ReFacto®/Xyntha®) remained comparable in the CS but not in the OS assay (Fig. 2). Thus, any plasma level of transgene-produced FVIII-SQ protein is expected to yield a similar CS activity as a continuous infusion of the same mass quantity of recombinant products (Sup. Fig. 7).

The diverging OS activities of transgene-produced and recombinant FVIII-SQ may be explained by differences between human and non-human production cells (Tab. 1). This could be due to differences in post-translational modifications[26] or protein folding, which is under investigation. Of note, other recombinant products, such as Fc-fused (Eloctate®) or unmodified (Nuwiq®) BDD-FVIII show lower OS than CS activity and yet are manufactured in human HEK 293 cells.[4,27,28] For a single-chain version of Fc-fused BDD-FVIII, the lower OS than CS activity is caused by delayed release from von Willebrand factor (VWF).[29] Therefore, additional modifiers (for example, during lyophilization) may influence assay discrepancies for recombinant products. Despite the discrepant activity measurements for transgene-produced FVIII-SQ, both OS and CS assay remain clinically meaningful to distinguish hemophilic from non-hemophilic conditions after administration of BMN 270 gene therapy (Fig. 5). Correlations between FVIII activity level and bleed rate were somewhat limited in our studies, since spontaneous bleeds were generally rare and self-reported by participants, and thus prone to some level of subjectivity. Nonetheless, the predicted absence of joint bleeds at $\geq$ 40 IU/dL median FVIII activity in both assays corresponds with the WHO definition of HA.[22] These data are also aligned with reports on severe HA patients receiving tertiary prophylaxis with recombinant products: at 40 IU/dL FVIII activity, the predicted proportion of patients without spontaneous joint bleeds was 100% (95% CI: 94.25% to 100%).[30]

Protection from spontaneous bleeds by transgene-produced FVIII-SQ appeared to persist even at lower levels (Fig. 5); both assays reliably predicted a negligible bleed risk at FVIII activity $\geq$ 15 IU/dL, consistent with literature and clinical experience to date.[31,32] At levels < 15 IU/dL,

however, the OS assay was associated with higher predicted joint bleed frequency compared to the CS assay. Therefore, clinical phenotypes should be observed carefully during clinical practice to evaluate how transgene-produced FVIII-SQ activity aligns with the theoretical risk of breakthrough bleeds in severe (< 1 IU/dL), moderate (1-5 IU/dL), and mild (5-40 IU/dL) HA conditions.[22,31-34] It might still be conceivable that an accelerated onset of FXa generation mediated by transgene-produced FVIII-SQ in vitro – and detected in the OS assay – may support coagulation in vivo, in particular at lower levels. Nonetheless, the CS activity was chosen as a surrogate endpoint during BMN 270 clinical development to ensure the most conservative assessment of hemostatic efficacy. In addition, using CS activity enables direct comparability between AAV gene therapy and current standard of care, given the consistent specific FVIII activity in the CS assay across different drug modalities.

Assay discrepancies with higher OS than CS values also exist for other AAV-based gene therapies in HA using BDD-FVIII[35] and in hemophilia B using FIX-Padua (FIX^{R338L}).[36] The Padua mutation increases the specific activity of FIX, and thus kinetic issues related to an accelerated onset of the clotting reaction in the OS assay may play a role for FIX-Padua as well. A more general commonality between AAV gene therapies could be the use of codon-optimized vectors. In rare cases, codon optimization influences protein folding and biological activity;[20,21] however, studies herein showed that codon optimization was not responsible for higher OS than CS activity of FVIII-SQ from a human liver cell line (Tab. 1). It will be valuable to investigate codon optimizations for FIX[37] and FIX-Padua[36] in the future.

Another consideration for liver-directed AAV-FVIII gene therapies is that hepatocytes are an ectopic expression site for FVIII, since native FVIII is mainly produced in various types of endothelial cells, including liver sinusoidal cells.[38,39] While our studies excluded higher OS than CS activity of FVIII-SQ as a liver-specific phenomenon (Tab. 1), it remains possible that endothelial cells modify or secrete FVIII differently than hepatocytes. More work is required to understand molecular differences in intra- and extra-cellular FVIII processing, including post-translational modifications, in different human cell types. Of note, such mechanisms would not apply to assay discrepancies for liver-directed AAV-FIX gene therapies, because native FIX is also produced in hepatocytes.[40]

Historically, adherence to the like-*vs*.-like principle when determining FVIII activity in plasma samples using plasma-derived calibrators resulted in high agreement between OS and CS

assays.[41] With the advent of recombinant FVIII products, however, assay discrepancies began to occur, typically with lower OS than CS activity.[2-4,27,42] Assay discrepancies are also common for novel, modified recombinant FVIII/FIX products with extended half-life (EHL) (reviewed in:[43-45]). In general, potency assignments of EHL-FVIII products show fairly limited OS/CS assay discrepancy, but different APTT reagents may yield discrepant results.[28,46-50] For EHL-FIX products, OS/CS assay discrepancies can be quite pronounced.[13,51-54] This complexity has led to guiding recommendations for potency assignment of recombinant FVIII/FIX products.[55]

A probable root cause underlying many of these assay discrepancies is a deviation from the like-*vs.*-like principle, i.e., defined molecular differences between non-native FVIII/FIX analytes and native FVIII/FIX calibrators, leading to diverging behavior in the assays. This was biochemically corroborated for some non-native FIX products.[13,56] These studies suggested that departures from the like-*vs.*-like principle may occur more often in OS than in CS assays due to their shorter readout time. The findings reported herein for gene-therapy-derived FVIII-SQ appear to corroborate this vulnerability of OS assays and confirm the broad utility of CS assays for clinical monitoring of non-native FVIII analytes. In laboratories where only an OS assay is available, a thoroughly evaluated, laboratory-specific OS/CS conversion factor might be established for gene-therapy samples to interpolate the corresponding activity in the CS assay.

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AUTHORSHIP CONTRIBUTIONS

Contributions: S.Ro., S.T., M.R., S.Z., T.C., and C.V. designed experiments; S.Ro. performed the OS kinetics experiments; D.M. performed FVIII-SQ purifications; S.Ro., M.R., M.H., J.S., and C.V. performed data analyses and prepared figures; S.Ro. and C.V. wrote the manuscript; K.J.P., S.Ra., E.S., A.G., G.P., B.K., and S.Z. critically reviewed and/or edited the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare the following competing financial interests: S.Ro. is chairman of Rossix AB; S.T. and M.R. are employees and hold stocks of Laboratory Corporation of America® Holdings; S.Ro. and G.P. are consultants for BioMarin Pharmaceutical, Inc.; M.H., J.S., T.C., B.K., S.Z., and C.V. are employees and own stocks of BioMarin Pharmaceutical, Inc.

DATA SHARING: For original data, please contact the corresponding author.

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TABLES

Table 1. OS and CS Activity of Recombinant FVIII-SQ Variants

Production Cell Type	Recombinant FVIII-SQ Variant Tested	Clone / Production Lot / Medium Conditions	OS FVIII Activity (IU/dL)	CS FVIII Activity (IU/dL)	OS/CS Ratio
CHO	Leu1880 (Xyntha®-like)	Clone 4-E2	96.2	92.8	1.037
		Clone 1-A12	132.0	129.5	1.020
		Clone 1-A12, 1:10 retest	11.5	14.6	0.788
CHO	Phe1880 (BMN270-like)	Clone 5-A5	44.9	54.5	0.823
		Clone 2-B4	29.8	39.3	0.758
		Clone 1-F5	23.9	27.3	0.877
CHO[a]	Xyntha® (Wyeth/Pfizer)	Lot W05255	76.0	92.7	0.820
HepG2	FVIII-SQ (BioMarin)	Lot 1	128.0	61.0	2.098
		Lot 2	322.6	176.7	1.826
		Lot 3	71.5	35.2	2.031
HepG2	codon-optimized[b]	serum-free	179.1	136.0	1.317
		serum-containing	882.1[c]	456.8	1.931
HepG2	not codon-optimized	serum-free	5.0	3.8	1.316
		serum-containing	28.7	14.2	2.021
HEK 293	codon-optimized[b]	serum-containing	56.9	32.6	1.744

[a] Based on literature reference.[19]
[b] Same codon optimization as in BMN 270.
[c] Back-calculated result is above the upper reportable limit but raw data for the diluted test sample were obtained within the validated range of quantification.

FIGURE LEGENDS

Figure 1. Correlation between FVIII Activities Measured in OS and CS Assay.

FVIII activity data from OS and CS assays in BMN 270 clinical trials 270-201 (**A**), 270-301 (**B**), and in 20 healthy donors (**C**) were correlated using linear regression. Each data point represents FVIII activity data from one visit; multiple visits for the same participant are represented by the same colored symbol. The slope of the regression curve reflects the constant rate of change between OS and CS activity measurements.

Figure 2. Specific Activity of Recombinant and Transgene-Produced FVIII-SQ.

Recombinant FVIII-SQ (Xyntha®) was spiked into plasma from severe HA individuals to determine the specific activity (IU/mg) using the OS or CS assay **(A, B)**. Mean specific activity for transgene-produced FVIII-SQ in individual participants from BMN 270 clinical trial 270-201 was determined using the OS or CS assay **(C, D)**. Mean specific activity for transgene-produced FVIII-SQ in individual participants from BMN 270 clinical trial 270-301 was determined using the OS or CS assay **(E, F)**. Dashed lines in A, C, and E represent the specification range for specific activity reported for Xyntha® (5,500-9,900 IU/mg).[16] Dashed lines in B, D, and F represent the specification range for specific activity reported for ReFacto® (7,600-13,800 IU/mg).[6] Given the lower OS than CS activity of recombinant FVIII-SQ, the specification range depends on which assay is used for product labeling (OS for Xyntha®; CS for ReFacto®). Error bars in C-F represent intra-participant standard deviations.

Figure 3. Kinetics of FIXa, FXa, and FIIa Formation in the OS Assay.

Plasma samples containing transgene-produced FVIII-SQ (Gene Therapy Plasma), collected from 2 different participants treated with BMN 270, and plasma samples containing native FVIII (Normal Plasma, Diluted) with the same CS activity were compared in stop kinetics experiments in the OS assay. FIXa generation (**A, B**), FXa generation (**C, D**), and FIIa (thrombin) generation (**E, F**) were monitored over 60 seconds after addition of $CaCl_2$. Error bars represent standard deviations between replicate assessments.

Figure 4. FIXa-Triggered Thrombin Generation Assay.

Native FVIII in healthy donor samples (Normal Plasma, n = 40) was compared with transgene-produced FVIII-SQ in trial 270-301 (Gene Therapy Plasma, n = 25), after individual participants (n = 7) had reached non-hemophilic CS activity levels (≥ 40 IU/dL) between weeks 23-26. Box-and-whisker plots were generated for thrombin peak height (A), ETP (B), lag time (C), and time-to-peak (D). The horizontal line in the box represents the median, the box represents the Q1-Q3 inter-quartile range (IQR), and the whiskers indicate the minimum and maximum value that fell within Q1 - 1.5 x IQR and Q3 + 1.5 x IQR, respectively. Dashed horizontal lines represent the lower and upper limit of the normal reference range, respectively. *P* values represent significance levels from Mood's median test.

Figure 5. Correlation of Joint Bleed Frequency and FVIII Activity Levels.

For each participant, the number of treated joint bleeds per 4-week interval was correlated with the median FVIII activity within each interval, as measured using the OS (blue circles) or CS (red circles) assay in BMN 270 clinical trials 270-201 (**A**) and 270-301 (**B**). The predicted bleed frequency per 4-week interval was modeled by negative binomial regression for OS activity (blue solid line) and CS activity (red dashed line). Shaded colored areas represent the corresponding 95% CI. No joint bleeds were reported for FVIII activity > 50 IU/dL in either assay; while these data were included in the modeling, they are not shown here.

Figure 6. Illustration of Kinetic Bias Leading to Higher OS Than CS Activity of Transgene-Produced FVIII-SQ.

Transgene-produced FVIII-SQ accelerates early FXa formation compared to native FVIII, resulting in a small increase in FXa concentration (pM range). This leads to faster thrombin activation and clot formation in the OS assay, which may explain the higher OS measurements since this assay uses a kinetic endpoint (time to visible clot) within the first 1 to 2 minutes of the coagulation reaction. In contrast, the CS assay uses more dilute test samples and determines FXa concentrations after a longer, fixed incubation period of 5 minutes. At this point, FXa generation

has been exponentially amplified (nM range) and presumably remains unaffected by the minute differences that occurred earlier in the reaction. The different timing of assay readout therefore leads to a kinetic bias between OS and CS assay, whereby the shorter assay (OS) reports higher FVIII activity values. pM, picomolar (pmol/L); nM, nanomolar (nmol/L)

Figure 1

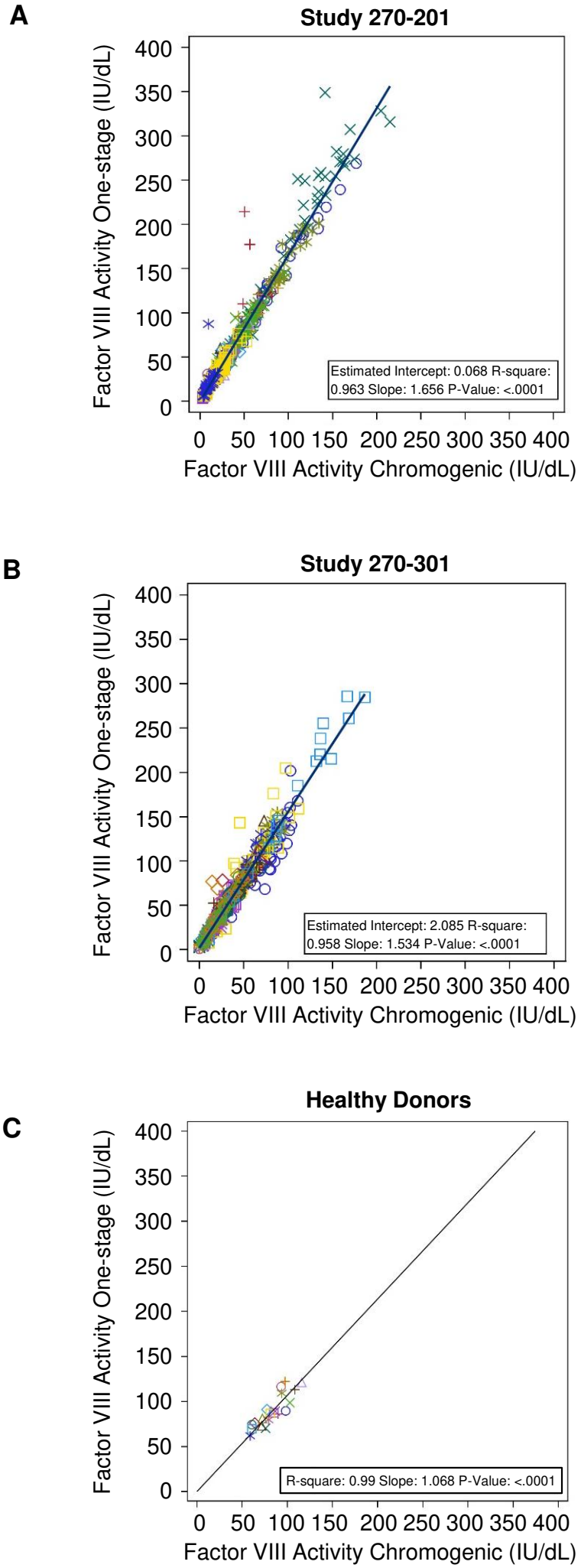

Figure 2

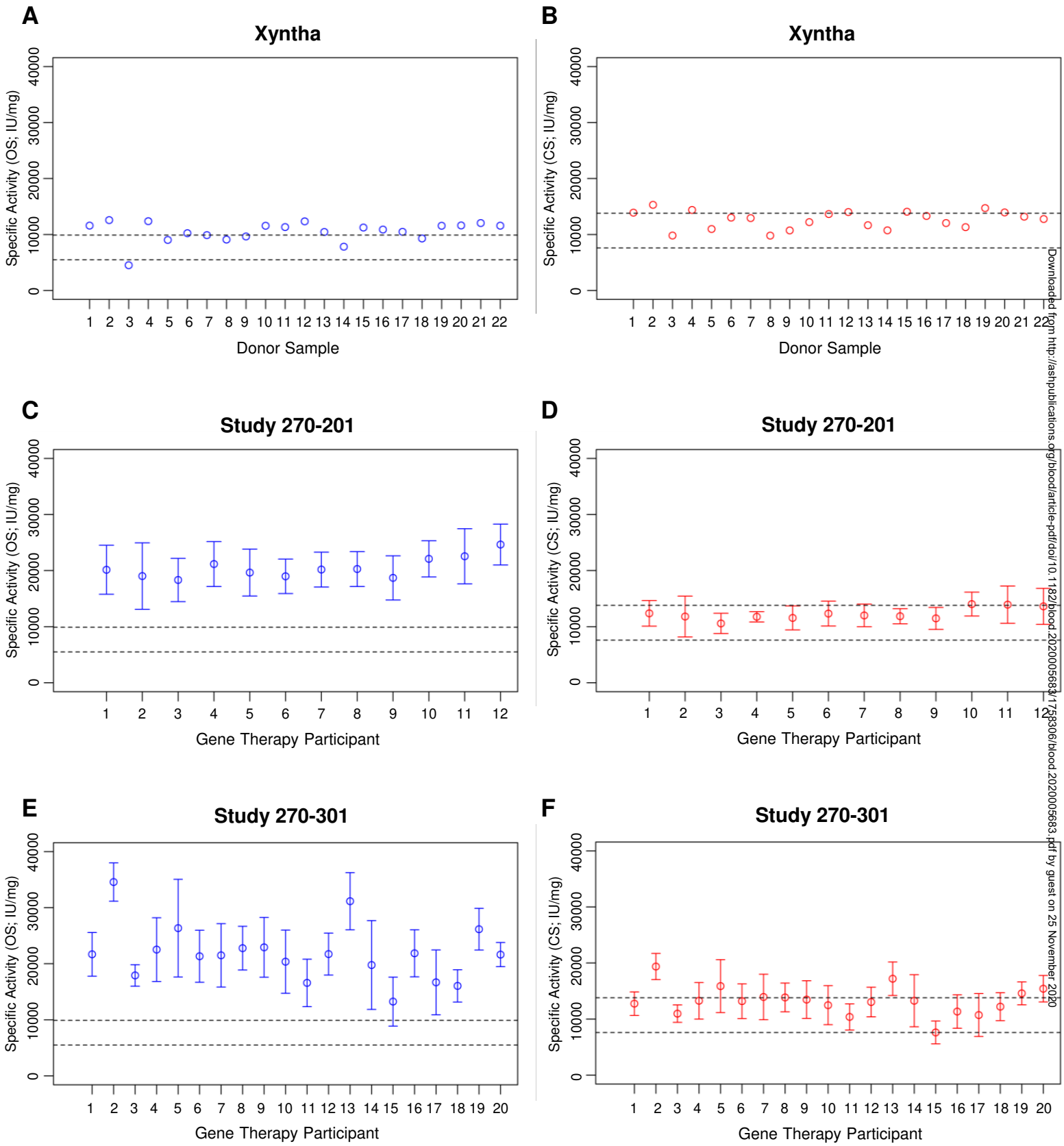

Figure 3

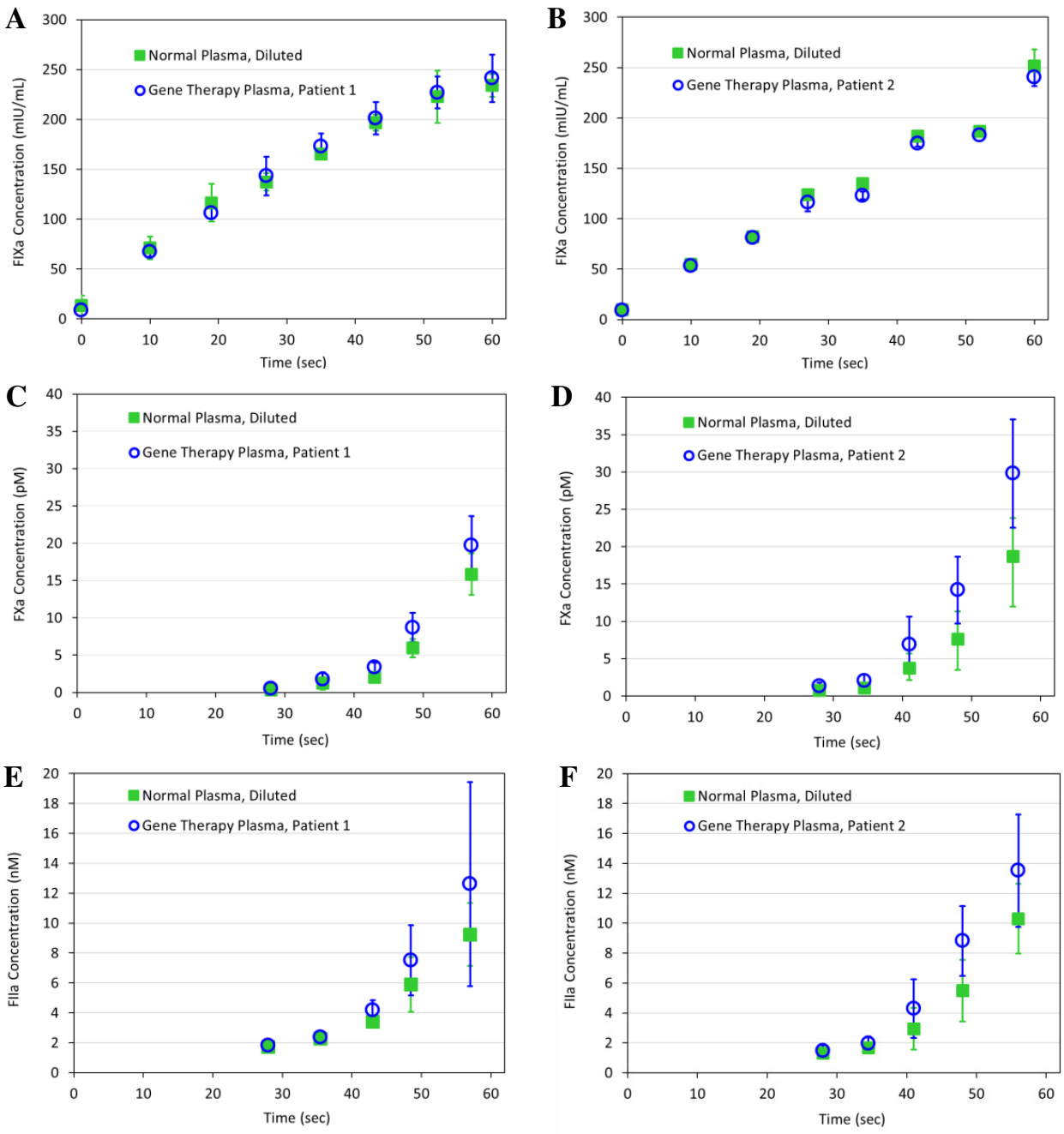

Figure 4

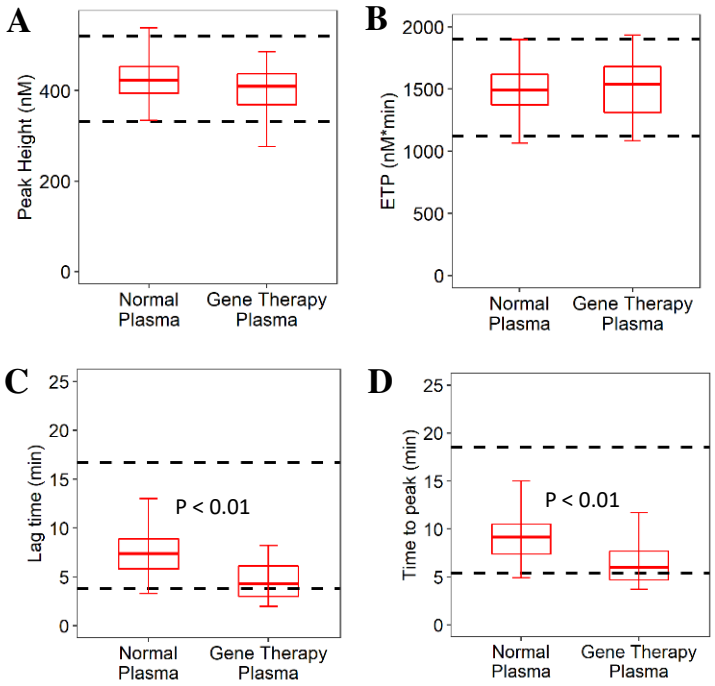

Figure 5

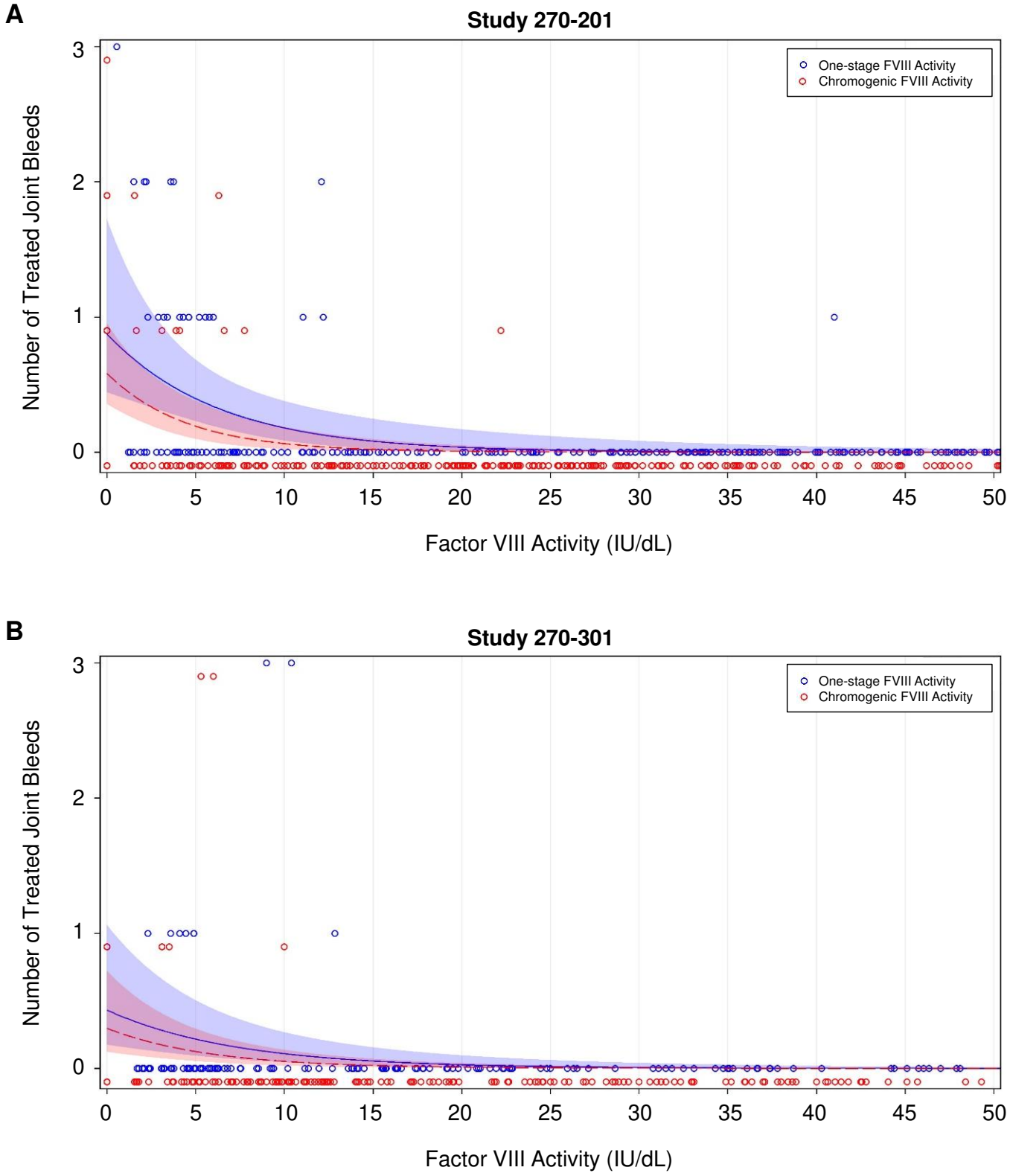

Figure 6