REGULAR ARTICLE

blood advances

Differences in wild-type– and R338L-tenase complex formation are at the root of R338L-factor IX assay discrepancies

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Key Points

- There was a threefold range in reported R338L-FIX activity in a field study of 15 different 1-stage (OSA) or chromogenic FIX:C assays (CSA).
- OSA discrepancies were linked to activating reagent/phospholipids, whereas FX influenced the relative potency of R338L-FIX in CSA.

Adeno-associated virus (AAV) gene therapy has the potential to functionally cure hemophilia B by restoring factor (F)IX concentrations into the normal range. Next-generation AAV therapies express a naturally occurring gain-of-function FIX variant, FIX-Padua (R338L-FIX), that increases FIX activity (FIX:C) by approximately eightfold compared with wild-type FIX (FIX-WT). Previous studies have shown that R338L-FIX activity varies dramatically across different clinical FIX:C assays, which complicates the monitoring and management of patients. To better understand mechanisms that contribute to R338L-FIX assay discrepancies, we characterized the performance of R338L-FIX in 13 1-stage clotting assays (OSAs) and 2 chromogenic substrate assays (CSAs) in a global field study. This study produced the largest R338L-FIX assay dataset to date and confirmed that clinical FIX:C assay results vary over threefold. Both phospholipid and activating reagents play a role in OSA discrepancies. CSA generated the most divergent FIX:C results. Manipulation of FIX:C CSA kits demonstrated that specific activity gains for R338L-FIX were most profound at lower FIX:C concentrations and that these effects were enhanced during the early phases of FXa generation. Supplementing FX into CSA had the effect of dampening FIX-WT activity relative to R338L-FIX activity, suggesting that FX impairs WT tenase formation to a greater extent than R338L-FIX tenase. Our data describe the scale of R338L-FIX assay discrepancies and provide insights into the causative mechanisms that will help establish best practices for the measurement of R338L-FIX activity in patients after gene therapy.

Introduction

Management of patients with hemophilia B has undergone a revolution in the last decade, and rapid innovation continues.[1,2] Factor (F)IX replacement with plasma-derived (pd), recombinant (r), and extended half-life FIX is the mainstay of treatment for bleeding episodes and prophylaxis in patients with hemophilia B. However, even with tailored and frequent dosing of FIX replacement products, patients experience low trough FIX activity (FIX:C) levels, which makes them prone to bleeding.[3] The risk of bleeding is especially high in joints that have been irreparably damaged because of imperfect management with FIX replacement products over many years.

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The full-text version of this article contains a data supplement.

Multiple adeno-associated virus (AAV)-based gene therapies are in development for the treatment of hemophilia B. Early versions of AAV gene therapy expressing the wild-type FIX (FIX-WT) protein showed that expression is stable and durable over 7 years of follow-up, reducing the frequency and severity of bleeds as well as the requirement for FIX replacement therapy.[4-6] Hemophilia B patients treated with early AAV gene therapies showed stable FIX activity levels of up to 12 IU/dL FIX:C, which is consistent with a mild bleeding phenotype.[4,7] Next-generation AAV vectors have an improved tropism for the liver and also incorporate a gain-of-function mutation in the FIX protein (FIX-Padua [R338L-FIX]) that confers a higher specific activity than FIX-WT.[8-10] FIX-Padua was first described in a family with thrombophilia,[11] and incorporation of the R338L-FIX variant into AAV gene therapy vectors has increased their potency by a reported eightfold compared with similar vectors expressing FIX-WT.[12,13]

The assignment of FIX potency for licensed FIX replacement products is measured by 1-stage activated partial thromboplastin time (aPTT)-based clotting methods calibrated against international concentrate standards (pdFIX). However, lower potency estimates are often obtained when rFIX products[14,15] are assayed using commercially available chromogenic assays. Similar to discrepancies reported for rFIX products when assayed against FIX plasma standards, there are discrepancies among 1-stage FIX clotting assays (OSA) and between OSA and chromogenic substrate assay (CSA) results when evaluating the R338L-FIX variant.[16,17] Since a variety of assays are being used in the clinic as a surrogate for R338L-FIX efficacy, it is critical that we gain a better understanding of what causes FIX assay discrepancies with the R338L-FIX variant to ensure that patients who receive AAV-R338L-FIX gene therapy products can be effectively monitored and managed.

We conducted a global, multicenter field study to characterize the degree of FIX assay discrepancy when measuring rR338L-FIX activity (rR338L-FIX:C) vs plasma standards using routine OSA and CSA and subsequently conducted a root-cause analysis to better understand the mechanisms behind assay discrepancies. To assess how our results may apply in the clinical setting, we indexed field study results to SynthASil FIX:C OSA levels and generated FIX:C ratios for all remaining FIX:C assays in the field study. We compared these ratios with a limited number of samples from clinical studies evaluating 2 different AAV-R338L-FIX gene therapy products. This enabled us to determine whether rR338L-FIX is a suitable surrogate for liver-expressed R338L-FIX when assessing the mechanisms of discrepant assay results.

Methods

FIX-Padua field study

Recombinant rR338L-FIX (*see* supplemental Materials and Methods and supplemental Figure 1 in the data supplement) was spiked into pooled inhibitor-free congenital hemophilia B patient plasma (<1 IU/dL FIX:C) to achieve nominal FIX activity levels of 5, 20, 50, 100, and 150 IU/dL as measured by the SynthASil FIX OSA, which was calibrated with the SSC/ISTH (Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis) Secondary Coagulation Standard Lot #4. These 5 rR338L-FIX samples plus 2 FIX control samples were sent to 38 laboratories in 11 countries (supplemental Figure 2). The 2 control samples used were the SSC/ISTH secondary coagulation factor plasma standard (lot 4; 105 IU/dL FIX:C) and the plasma-derived WHO (World Health Organization) Fifth International Standard (IS) for FIX Concentrate (NIBSC [National Institute for Biological Standards and Control] code: 14/148) spiked at 126 IU/dL into the same congenital hemophilia B plasma. Across the 38 laboratories, 13 different types of OSAs and 2 CSAs were performed to characterize FIX assay discrepancies. Each laboratory performed its routine FIX assays on 3 different occasions, using the 5 rR338L-FIX samples and 2 control samples. Laboratories were blinded to FIX:C concentrations in the samples. The countries represented in the field study, as well as the FIX assays, are shown in Table 1. The rR338L-FIX field study methodology is described in detail in supplemental Materials and Methods in the data supplement.

FIX assays

OSA and CSA were performed according to the manufacturer's instructions, except in mechanistic studies where assays were supplemented with pdFX or rFVIII or the activation time was altered. To achieve the desired FX or FVIII concentration in certain experiments, supplementary FX or FVIII was added directly to the chromogenic assay kit reagent containing the protein of interest.

Clinical samples

Thirty-three citrated blood samples were collected at various times from 5 patients who participated in the FLT180a (AAVS3-FIX-Padua) phase 1/2 trial (ClinicalTrials.gov Identifier: NCT03369444). Blood samples were processed to plasma before being stored at −80°C. SynthASil FIX OSA and ROX FIX activity assays were performed at the Royal Free Hospital in London, United Kingdom. The patients were local to the Royal Free Hospital and provided informed written consent. The field study results were compared with FIX:C results from another AAV-R338L-FIX clinical study[17] via the extraction of published clinical data.

FXI activation by OSA FIX assay activators

A system of purified contact pathway proteins was used to examine the potency of each OSA activator in generating FXIa. To enable comparisons between aPTT reagents in our purified system, we regulated the reactions by omitting prekallikrein/kallikrein. Instead, we included 2-chain high molecular weight kininogen (TC-HMWK) to facilitate the interaction between FXIIa and FXI on the negatively charged aPTT reagent surfaces. The 6 aPTT reagents tested were silica- (TriniCLOT aPTT S, TriniCLOT aPTT HS, and SynthASil), ellagic acid- (ACTIN-FS and SynthAFax), or polyphenol-based (Cephascreen). Briefly, plasma-derived purified contact pathway proteins at their normal plasma concentration (400 nM FXII, 600 nM TC-HMWK, 30 nM FXI) were incubated with each of the 1-stage activating reagents. FXIa activity was quantified against a pdFXIa standard using the chromogenic substrate S-2366 (2.1 mM).

Results

R338L-FIX field study

AAV gene therapies expressing the R338L-FIX variant aim to produce FIX:C activity levels that approach, or are within, the normal range of FIX activity observed in healthy individuals (typically 50 to 150 IU/dL FIX). Therefore, the most clinically relevant activity levels for assessing R338L-FIX assay discrepancies are across the mild hemophilia B and normal range of FIX:C (5 to 150 IU/dL FIX).

Table 1. Assay kits used by the participating laboratories in each country and corresponding interlaboratory variability

Assay kit	Australia	Canada	Czech Republic	France	Germany	Italy	The Netherlands	South Africa	Sweden	United Kingdom	United States	Total Labs	Interlaboratory CV (%)*		
													rR338L-FIX	WHO (Fifth IS)	SSC lot #4
PTT Automate	1	–	–	1	–	–	–	1	–	–	–	3	10	2	5
TriniCLOT aPTT S	1	–	–	–	–	–	–	–	–	–	–	1	1*	2*	2*
TriniCLOT aPTT HS	–	–	–	1	–	–	–	–	–	2	–	3	7	6	8
Cephascreen	–	–	1	1	–	–	1	–	–	–	–	3	17	8	12
APTT-SP	1	–	–	–	–	–	–	–	–	2	–	3	13	16	25
PPT-LA	–	–	–	–	–	–	1	–	–	–	–	1	18*	19*	21*
CK Prest	–	–	–	3	–	–	–	–	–	1	–	4	3	5	4
SynthASil	1	1	–	1	–	1	–	–	–	3	3	10	**6**	**11**	**8**
ACTIN-FS	2	2	–	1	–	–	–	–	–	6	–	11	11	11	11
Actin	–	–	–	–	–	–	–	–	–	1	–	1	11*	8*	6*
Pathromtin	–	–	3	–	1	–	–	–	–	3	–	7	7	10	11
SynthAFax	–	2	–	–	–	–	–	–	–	7	1	10	11	8	7
Actin-FSL	1	–	1	–	–	–	–	–	–	3	2	7	9	7	11
ROX FIX (Rossix)†	–	1	–	1	1	1	–	–	1	4	2	11	23	13	9
Biophen FIX (Hyphen)†	1	1	1	1	–	–	1	–	–	3	–	8	15	8	7

CV, coefficient of variation; FIX, factor IX; FIX:C, FIX activity; IS, International Standard; r, recombinant; SSC, Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis; WHO, World Health Organization.

CV% in these cases are intralaboratory CV.

*Denotes assays that were only performed at 1 laboratory.

†Highlights chromogenic FIX:C assays.

The results of this field study showed that R338L-FIX assay discrepancies were consistent for all OSA and CSA across the 5 to 150 IU/dL FIX:C range (Figure 1A); there was a 1.8-fold variation across OSA results (Actin-FSL vs PTT Automate) and a threefold variation was observed when comparing OSA and CSA (Figure 1B). In general, we found that particulate activators (eg, silica and kaolin) produced higher R338L-FIX:C results than ellagic acid and that the phospholipid source had a dramatic impact on the observed R338L-FIX:C concentrations (eg, Actin FS vs Actin FSL) (Figure 1B and supplemental Table 1). Since the results from all FIX OSA were nearly perfectly correlated (Pearson $r > 0.99$; $P < .001$), they can be described by simple linear regression. This makes it possible to calculate FIX:C ratios that enable the prediction of R338L-FIX:C results from various assay methods using a single measured value (supplemental Table 2).

Interlaboratory R338L-FIX assay variability

Enrollment of a broad range of centers enabled us to capture interlaboratory variability for most assays. Table 1 shows interlaboratory variability (or intralaboratory when data were collected from only 1 laboratory) for pdFIX standards (WHO Fifth IS and SSC Lot 4) and the nominal 100 IU/dL R338L-FIX sample. Interlaboratory variability (CV%) was similar for rR338L-FIX ("nominal 100 IU/dL" FIX:C, CV = 3% to 23%) and pdFIX standards (WHO Fifth IS: 126 IU/dL FIX:C, CV = 2% to 16%; and SSC Lot 4: 105 IU/dL FIX:C, CV = 4% to 25%). There was a trend toward increased variability in R338L-FIX samples with decreased FIX:C (supplemental Figure 3), which is consistent with published results evaluating other FIX products and plasma-derived standards.[14,15]

Generalizing field study results to liver/AAV gene therapy expressed R338L-FIX

The volume of plasma required to assess R338L-FIX activity (supplemental Materials & Methods and supplemental Figure 2) precluded the use of plasma from FLT180a clinical trial patients in the field study. Therefore, to compare the activity of R338L-FIX expressed in clinical trial patients who received FLT180a with

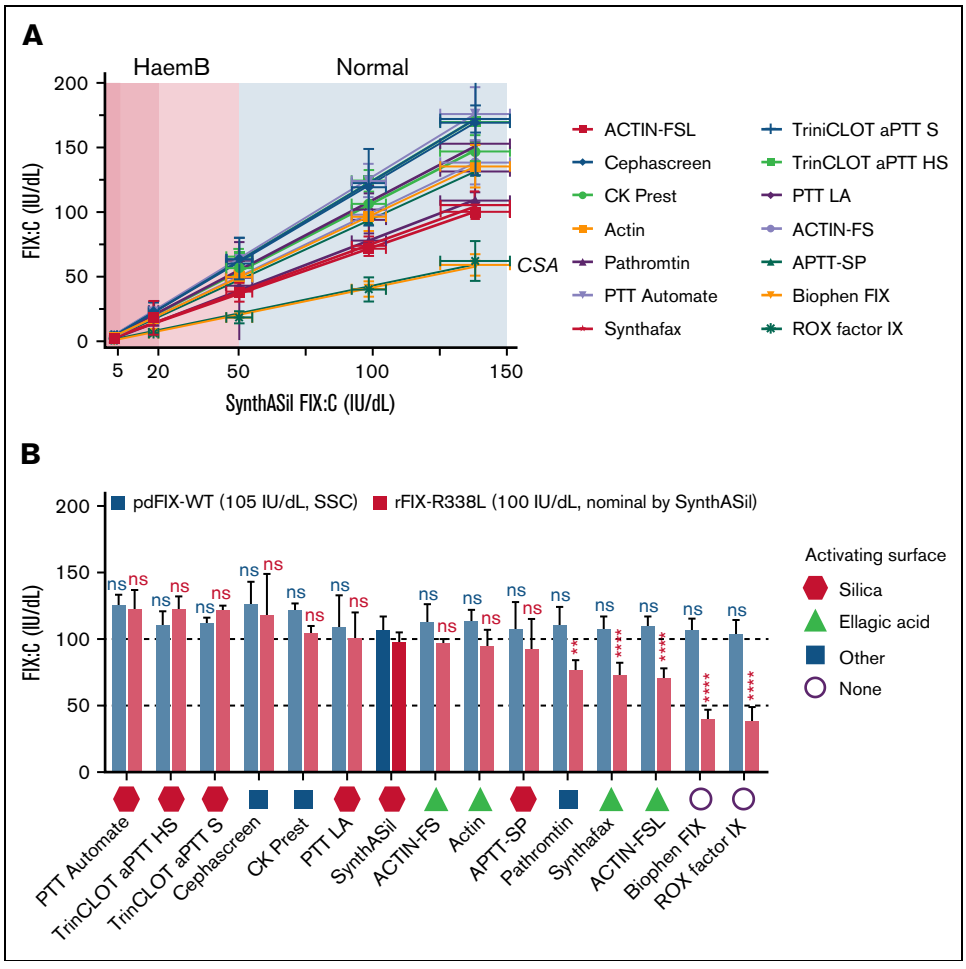


Figure 1. Summary of field study data compared with average SynthASil OSA results. (A) R338L-FIX:C of field study samples measured by the SynthASil OSA plotted against the values in the same samples measured by 13 different OSAs and 2 CSAs against a hemophilia B (red) or normal (blue) background. (B) Comparison of FIX activity in plasma samples spiked with normal pdFIX-WT (SSC Lot 4, 105 IU/dL FIX:C) and rR338L-FIX (100 IU/dL nominal activity by SynthASil) across 13 OSAs and 2 CSAs. Symbols indicate the activating surface corresponding to each assay. Error bars indicate interlaboratory standard deviation; all linear regression fits, $R^2 > 0.98$. Mean differences between any 2 assays were compared separately for pdFIX-WT and rR338L-FIX using Tukey's multiple comparison adjustment with an overall type I error rate of 0.05. Only comparisons to SynthASil are shown. $^{**}P < .01$, and $^{****}P < .0001$. ns, not significant.

rR338L-FIX control material used in the field study, we measured FIX:C in 33 FLT180a clinical samples from 5 patients using the SynthASil OSA and ROX FIX CSA methods. The discrepancy in FIX activity measured using SynthASil OSA and ROX FIX CSA was broadly similar between the FLT180a clinical trial patients and the 5 rR338L-FIX–spiked field study samples (Figure 2A). We also used the field study data to predict ROX FIX assay results based on SynthASil FIX OSA inputs (supplemental Table 2) and compared the ROX FIX results with empirical data (Figure 2B). The predicted and empirical ROX FIX data were strongly correlated, with <10% CV between these methods. Conversion of published SynthASil FIX:C values for AAV/liver-expressed R338L-FIX[17] to ROX FIX, Actin FSL, PTT Automate, and CK Prest also produced results in close agreement with the respective measured values (Figure 3). The similar degree of assay discrepancy between various OSA and ROX FIX CSA in the field study and clinical samples (Figures 2A and 3) validates our decision to use recombinant protein in the field study. This similarity also demonstrates that rR338L-FIX is an appropriate tool for evaluating the biochemical mechanisms that drive discrepancies between FIX assay results.

Mechanistic assessment of FIX assay discrepancies

To better understand the mechanisms causing the FIX assay discrepancies observed in the field study, we built upon work that characterized the enzymology of R338L-FIX. Kinetic experiments by Samelson-Jones and colleagues[13] demonstrated that the R338L-FIX tenase complex (R338L-FIXa-FVIIIa) has a threefold higher turnover (k_{cat}) of FX compared with FIX-WT tenase, whereas the R338L-FIX variant showed little if any improvement relative to FIX-WT in the absence of FVIIIa or when the FVIII mimetic emicizumab was used. These findings indicate that a likely source of assay discrepancy may relate to differences in the WT and R338L tenase reaction in OSA and CSA.

We initially hypothesized that the apparent lower R338L-FIX activity reported by CSA could be related to the prolonged reaction time (8 min for ROX FIX and 3 min for Biophen FIX) compared with the typical OSA (30 to 120 s clot times). To determine the effect of reaction time on FIX:C values, we measured and matched rR338L-FIX and rFIX-WT samples with SynthASil OSA rFIX:C of 50, 100, and 150 IU/dL in parallel with pdFIX-WT samples as control samples in shortened (3 min) and prolonged (8 min) activation reactions. Interestingly, in contrast with the typical 8-minute reaction in the ROX FIX kit (supplemental Figure 4A), the activity of rR338L-FIX in the 3-minute reaction was greater than that of the matched rFIX-WT sample (supplemental Figure 4B), translating into increased apparent activity (Figure 4B). Prolonging the reaction time in the Biophen FIX kit did not result in any changes compared with standard methods (Figure 4D).

We also hypothesized that the components of the assay reaction, including enzyme (R338L-FIX vs FIX-WT), cofactor (FVIII), or substrate (FX), could influence the activity of the R338L-tenase. To determine the impact of tenase components on R338L-FIX activity observed in each kit, we calculated FIX concentrations based on a known spike concentration and assayed the amount of FXIa, FVIII, and FX in the reaction of both CSAs evaluated in the field study (supplemental Table 3). Consistent with the typical description of FIX assays, both FVIII and FX were in excess of FIX after sample dilution. FXIa concentration was lower in ROX FIX (1 nM) than in Biophen FIX (5 nM), suggesting slower FIX activation. The amount of FVIII was close to the reported physiological value of 0.7 to 2 nM[18] (0.5 nM in ROX FIX and 1.5 nM in Biophen FIX), whereas the amount of FX in these reactions was 34 nM (ROX FIX) and 61 nM (Biophen FIX), which is three- to sixfold lower than plasma FX concentrations.

To determine whether FX is rate limiting when measuring R338L-FIX:C in FIX CSA, we spiked the FX-containing reagent in each kit with additional FX to a final concentration of 180 nM and measured R338L-FIX or FIX-WT activity. Unexpectedly, increasing the FX concentration reduced the amount of FXa generated in the reaction, yielding lower absorbance values for all samples, but to a greater extent for rFIX-WT than activity-matched rR338L-FIX samples (data not shown). These differences did not change the interpolated FIX-WT:C values since the standard curves generated with pdFIX-WT (Figure 5A,C) were similarly impacted as rFIX-WT samples. However, this had the effect of increasing the inferred value of R338L-FIX:C relative to FIX-WT:C (Figure 5B,D). A FX concentration of 460 nM magnified this effect in the Biophen FIX

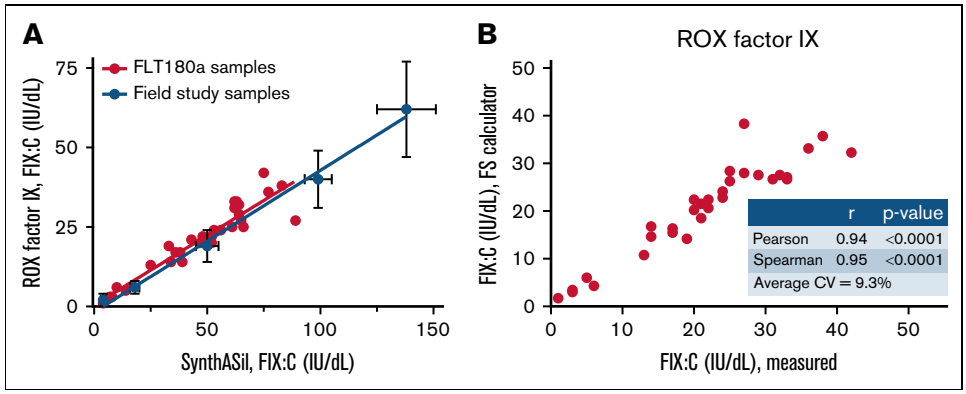
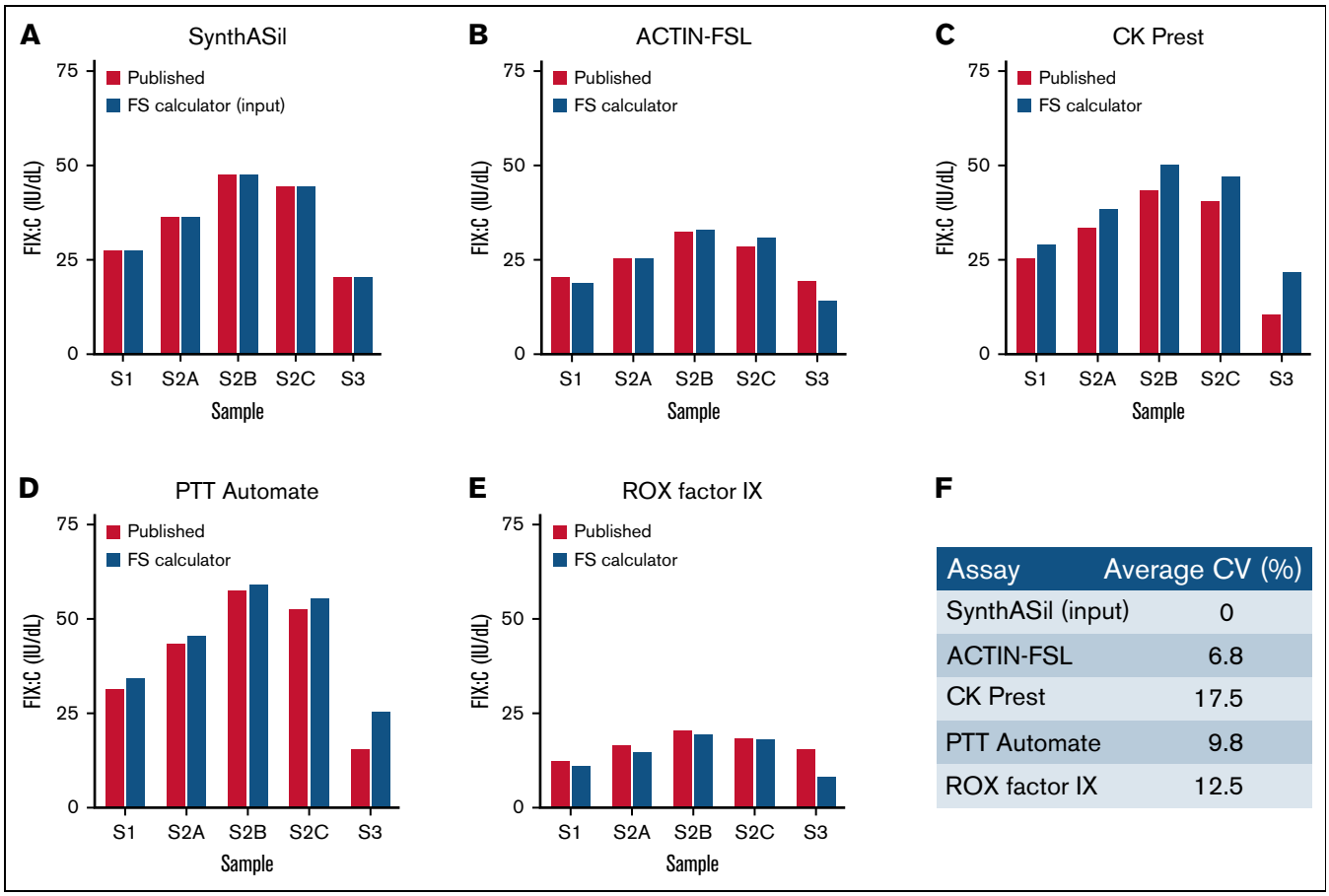


Figure 2. Comparison of pdR338L-FIX expressed by FLT180a-treated patients and rR338L-FIX. (A) R338L-FIX:C in plasma samples collected from FLT180a-treated patients was measured using both the SynthASil OSA and ROX FIX CSA methods. The resulting values were compared with the activity of rR338L-FIX spiked in hemophilia B plasma at 5, 20, 50, 100, and 150 IU/dL FIX:C, determined by the SynthASil OSA FIX method. (B) Empirical SynthASil OSA FIX:C results were used in conjunction with FIX:C ratios (derived from supplemental Table 2) to estimate ROX FIX:C results, which are directly compared with empirical ROX FIX:C values. FS, field study.



Assay	Average CV (%)
SynthASil (input)	0
ACTIN-FSL	6.8
CK Prest	17.5
PTT Automate	9.8
ROX factor IX	12.5

Figure 3. Analysis of published R338L-FIX assay results using the field study calculator. Published FIX:C SynthASil assay data for 4 R338L-FIX gene therapy patient samples were used as input (A) in the field study calculator, and the predicted values for (B) ACTIN-FSL, (C) CK Prest, (D) PTT Automate, and (E) ROX FIX were compared with the matching assay-measured values. The average CV (%) between measured and predicted values is shown in (F).

CSA (supplemental Figure 5). Similarly, we determined that FVIII supplementation had only a minor impact on FXa generation in CSA (absorbance values, data not shown). In both assays, an increase in FVIII concentration resulted in R338L-FIX:C values that were typically decreased to a greater extent than those observed for FIX-WT (supplemental Figure 6).

FXI activation by 1-stage FIX assay activators

The field study data implied that the composition of the activating reagent contributes to the R338L-FIX assay discrepancy. We hypothesized that R338L-FIX activity determined by OSA might be related to the amount of FXIa generated during the OSA reaction. In a purified system, ellagic acid-based (SynthAFax and Actin-FS) and polyphenol (Cephascreen) reagents appeared to generate more FXIa activity than silica-based reagents (TrinCLOT aPTT S, TrinCLOT aPTT HS, and SynthASil) (supplemental Figure 7A). Since most aPTT reagents make use of different proprietary phospholipids, it is difficult to decouple differences in FXIa and how phospholipids impact R338L-FIX assay discrepancies, but the impact of the negative surface reagent on FXIa activity is discernible. In a similar purified system with FXIa instead of FXII, there were clear differences in FXIa activity with the various activator/phospholipid reagents (supplemental Figure 7B), suggesting that the activator and/or phospholipid present impact on FXIa generation and activity.

Discussion

Our R338L-FIX field study is the first comprehensive characterization of the performance and variability of routine FIX OSA and CSA in measuring rR338L-FIX:C. Our data demonstrate that FIX:C results for R338L-FIX can vary over threefold, with the OSA PTT Automate and TrinCLOT aPTT S and HS yielding the highest results, and CSA ROX FIX and Biophen FIX yielding the lowest results. The study also showed that interlaboratory variability was similar for R338L-FIX and pdFIX across all assays evaluated. As R338L-FIX activity decreased, assay variability increased, which is in line with results from other field studies that characterized novel FIX products.[14,15] Collectively, these data suggest that R338L-FIX assay variability is likely because of well-established issues of assay precision rather than inexperience with a particular assay or FIX product.

Discrepancies in R338L-FIX:C measurements between OSA can be explained in part by differences in the activating surfaces and phospholipids, but the mechanisms underlying CSA vs OSA discrepancies are more complex. To investigate these, we carried out FX spiking experiments in both CSA kits and observed that excess

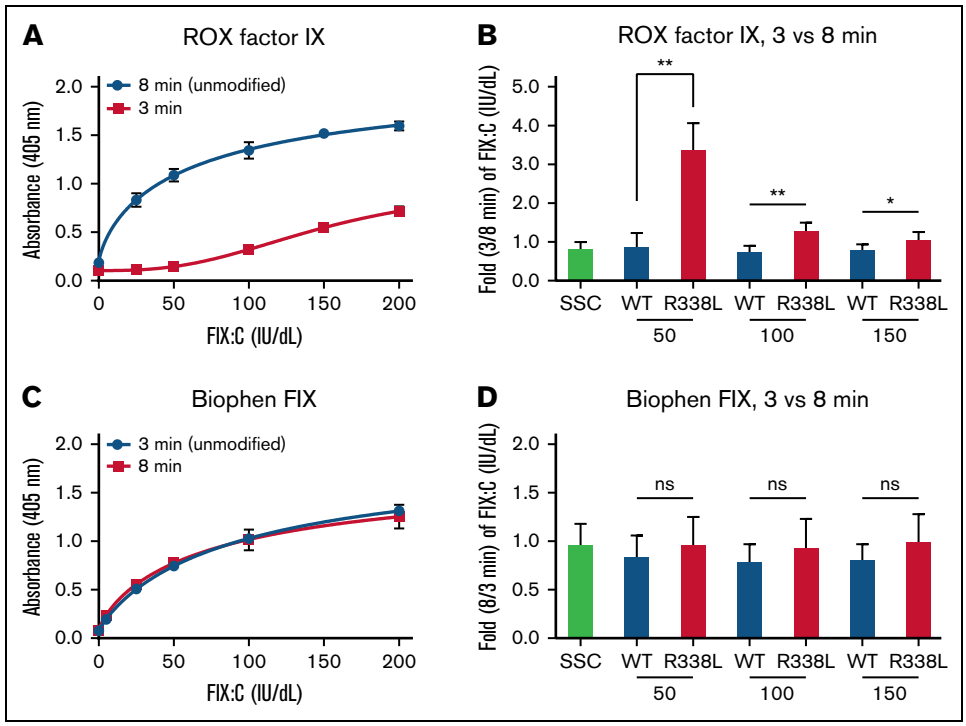


Figure 4. Effect of activation time on R338L-FIX activity in chromogenic FIX assays. rFIX-WT and rR338L-FIX spiked samples with 50, 100, and 150 IU/dL FIX:C by SynthASil OSA assay were tested alongside SSC Lot 4. Representative standard curves (A) ROX FIX kit and (B) fold change (3 min/8 min) in FIX activity of 3 minutes and standard protocol (8 min) assay reactions. Representative standard curves (C) Biophen FIX kit and (D) fold change (8 min/3 min) in FIX activity for 8 minutes and standard protocol (3 min) assay reactions. Standard curves are fitted with a 4-parameter logistic regression model. Values are mean + SD of ≥3 independent experiments. Means were compared using a paired 2-tailed *t* test. *$P < .05$ and **$P < .01$. ns, not significant; SD, standard deviation; SSC, Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis.

FX paradoxically impaired FXa generation. This suppression was relatively greater for FIX-WT compared with R338L-FIX, resulting in an approximately twofold increase in R338L-FIX activity compared with FIX-WT. A closer examination of how FVIIIa is generated in a generic OSA and the ROX FIX CSA provides a plausible explanation of why the ROX FIX CSA is more sensitive to FX than the OSA; put simply, FVIIIa is generated before FIXa in OSA[19,20] ("early FVIIIa"), whereas FIXa generation precedes FVIIIa in the ROX FIX CSA ("late FVIIIa"). When FVIIIa is absent or its activation is delayed, as in the ROX FIX CSA, nascent FIXa can form a relatively unproductive complex with FX, temporarily preventing FIXa from forming a productive tenase complex as FVIIIa is generated. Since limited FVIIIa is present before FIXa generation in the OSA, FIXa would be less prone to forming unproductive complexes with FX. Samelson-Jones and colleagues[13] observed a similar phenomenon in the OSA but the R338L-FIX activity boost was much less pronounced with only a 20% to 25% increase with supplemental FX, indicating that these events are also likely taking place in OSA reactions.

Our interpretation of FX supplementation experiments suggests that the dynamics of FVIII activation are at the root of R338L-FIX assay discrepancies. Earlier studies have traced FVIII activation to tissue factor–FVIIa complexes formed in the earliest stages of coagulation.[21,22] With this evidence, it is tempting to conclude that OSAs, with their "early FVIIIa," more accurately represent FIX activity when assessing the R338L-FIX variant. However, the timing and relative abundance of FVIIIa compared with FIXa during other phases of coagulation are also known to be important for hemostasis. Ultimately, a large clinical dataset will be needed to confirm which assay best corresponds to bleeding phenotypes in patients treated with liver-directed AAV gene therapy containing the R338L-FIX variant. Consistent with these early events in the ROX FIX assay reaction, reduction of the reaction time to 3 minutes also magnifies the difference between R338L-FIX and FIX-WT activities when compared with the stock 8-minute reaction (Figure 4), while FVIII supplementation had little overall impact on FIX activity (supplemental Figure 6). We cannot fully explain why supplemental FX differentially impacts FIX-WT and R338L-FIX activity in the ROX FIX CSA, though the increased affinity of FX to FIXa-WT compared with R338L-FIX is likely a contributing factor. Notably, we also found that the impact of supplemental FX in the Biophen CSA was intermediate to the OSA and ROX FIX assays, which was also expected given that the formation of FIXa and FVIIIa occurs in parallel in the Biophen CSA.

We acknowledge that the use of rR338L-FIX as a surrogate for AAV/liver-expressed R338L-FIX is a limitation of this study, but preliminary data using multiple FIX assays suggest that rR338L-FIX results are generalizable to R338L-FIX concentrations observed with investigational AAV-R338L-FIX gene therapy products (Figures 2 and 3). Although R338L-FIX expressed from different products may have different properties,[23] the comparability of R338L-FIX assay discrepancies from 2 AAV gene therapy products

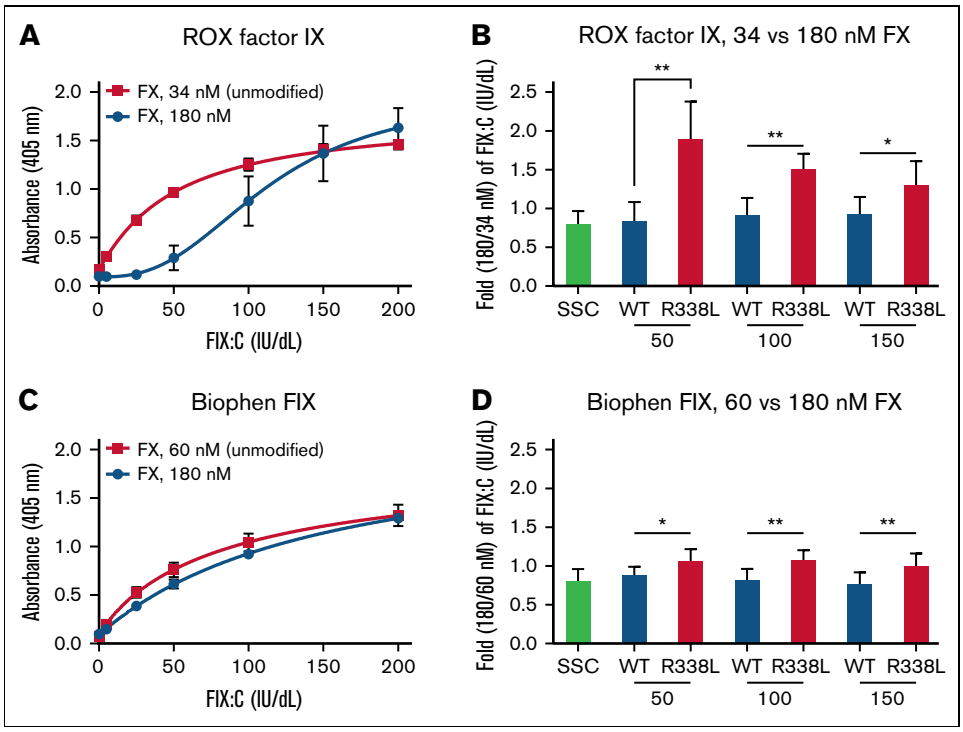


Figure 5. Supplemental FX causes an apparent increase in R338L-FIX activity in chromogenic FIX assays. rFIX-WT and rR338L-FIX spiked samples with 50, 100, and 150 IU/dL FIX:C by SynthASil OSA were tested alongside SSC Lot 4. ROX FIX and Biophen FIX assays were performed according to the standard protocol or with FX supplemented to 180 nM. (A) ROX FIX representative standard curves; (B) fold changes (180 nM/34 nM FX) of interpolated FIX activity; (C) Biophen FIX representative standard curves; and (D) fold changes (180 nM/60 nM FX) of interpolated FIX activity. Standard curves are fitted with a 4-parameter logistic regression model. Values are mean ± SD, n ≥3 independent experiments. Means were compared using a paired 2-tailed *t* test. **P* < .05 and ***P* < .01. ns, not significant; SSC, Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis.

implies that these differences do not alter routine clinical FIX assay results. Our study was comprehensive, given that the OSAs included represent 90% of the FIX:C assay reagents currently used in CAP (College of American Pathologists)-accredited laboratories in the United States, as per the 2014 CAP Proficiency Survey.[17] The assays evaluated also covered available FIX CSA and the different types of aPTT activating reagents used in OSA. However, additional work with clinical samples is needed to confirm the generalizability of our findings and to determine whether R338L-FIX expressed from different AAV gene therapy products performs similarly in clinical FIX assays.

Conclusions

In summary, we performed a global multicenter R338L-FIX field study to characterize the performance and variability of 15 routinely used clinical FIX assays. Our findings demonstrate that recombinant R338L-FIX protein is a suitable surrogate for AAV/liver-expressed R338L-FIX when evaluating the mechanisms driving discrepant results. We identified several mechanisms that contribute to FIX assay discrepancies between the R338L-FIX variant and plasma standards. Our data suggest that discrepancies among OSA results are caused by the activating reagent and/or reaction time, which all impact FXIa generation. Unlike OSA, the FIX assay discrepancy observed in the CSA appears to be a function of how FVIIIa is generated. Interestingly, although the ROX FIX and Biophen FIX CSA showed the same degree of assay discrepancy, this appeared to occur via a collection of different factor levels and mechanisms of tenase formation. We believe that these data will help inform the hemophilia community and treating physicians about assay discrepancy when measuring R338L-FIX activity and, with emerging annualized bleeding data, will help guide clinicians when managing patients with a given factor level in the event of traumatic bleeds or surgery.

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Authorship

Contribution: J.H.F., E.S., J.L., D.S., S.K., and A.C.N. conceived and/or designed the study; E.S., A.R., E.G., A.G., D.V., and I.-M.Y. performed experiments; J.H.F., E.S., A.R., E.G., A.G., D.V., and S.K. analyzed and interpreted the data; J.H.F., E.S., I.-M.Y., P.C., R.C., and A.C.N. wrote and/or revised the manuscript; D.S. and I.-M.Y. provided administrative, technical, or material support (reporting or organizing data, managing CRO); and all authors reviewed the manuscript.

Conflict-of-interest disclosure: J.H.F., E.S., A.G., I.-M.Y., D.V., J.L., D.S., and R.C. were or currently are employees of Freeline Therapeutics and are equity holders. J.H.F., E.S., A.C.N., and R.C. are inventors on patent rights filed by Freeline Therapeutics. A.R. has received honoraria from Novo Nordisk and Takeda. D.V. is an employee and equity holder for VarmX B.V. J.L. is employed by Corlieve Therapeutics, a fully owned subsidiary of uniQure N.V., and holds uniQure stock options. S.K. received consultancy fees from Freeline and Instrumentation Laboratory/Werfen. P.C. has served on advisory boards for Bayer, Boehringer Ingelheim, CSL Behring, Chugai, Freeline, Novo Nordisk, Pfizer, Roche, Sanofi, Spark, Sobi, and Takeda and has received research funding from Bayer, CSL Behring, Freeline, Novo Nordisk, Pfizer, SOBI, and Takeda. A.C.N. is an advisor to Freeline, is a founder of Freeline and NovalGen Therapeutics, holds equity in both these companies, and is an inventor on patent rights licensed to Freeline Therapeutics and NovalGen Therapeutics. E.G. declares no competing financial interests.

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