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Field Study and Correlative Studies of Factor IX Variant FIX-R338L in Participants Treated with Fidanacogene Elaparvovec

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Abstract

Background Fidanacogene elaparvovec, an adeno-associated virus-based gene therapy vector expressing the high-activity factor IX (FIX) variant FIX-R338L, is in development for hemophilia B. One-stage clotting (OS) assays and chromogenic substrate (CS) assays are commonly used to measure FIX-R338L variant activity. Data from ongoing trials suggest FIX activity varies between different OS and CS assays.

Material and Methods To better understand FIX-R338L activity in clinical samples, an international multisite field study was conducted across a central laboratory and 18 local laboratories, using standard protocols, reagents, and instrumentation, with individual participant samples from a phase 1/2a study of fidanacogene elaparvovec.

Results Unlike the wild-type FIX control, FIX-R338L activity was higher with the OS silica-based assay versus OS ellagic acid–based and CS assays. Variation in FIX activity was greater at the lowest activity levels. Activated FIX (FIXa) in plasma could result in higher OS assay activity or increased thrombin generation, which could overestimate FIX activity. However, FIXa was not detected in the participant samples, indicating that it was not contributing to the OS assay differences. Since individuals on gene therapy may receive exogenous replacement FIX products, replacement products were spiked into patient plasma samples to target a therapeutic concentration. Exogenous FIX was additive to endogenous FIX-R338L, with no interference from FIX-R338L.

Conclusion These results demonstrate FIX-R338L activity can be measured with OS and CS assays in clinical laboratories and provide insight into assay variability when measuring

Keywords
- biological assays
- blood coagulation tests
- factor IX
- gene therapy
- hemophilia B

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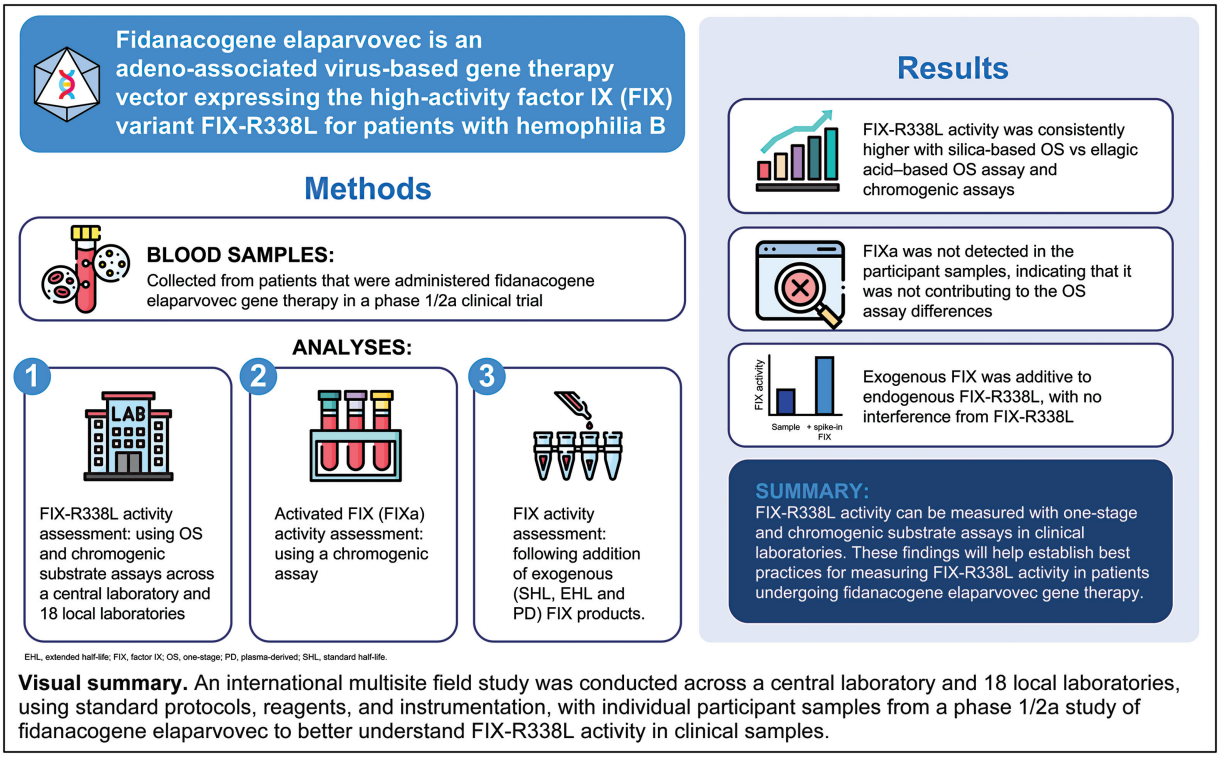

Visual summary. An international multisite field study was conducted across a central laboratory and 18 local laboratories, using standard protocols, reagents, and instrumentation, with individual participant samples from a phase 1/2a study of fidanacogene elaparvovec to better understand FIX-R338L activity in clinical samples.

FIX with endogenously produced FIX-R338L. The findings may help establish best practices for measuring FIX-R338L activity (Clinicaltrials.gov identifier: NCT02484092).

Introduction

Hemophilia B is an X-linked chromosomal disorder that results in a deficiency of coagulation factor IX (FIX).[1] Current management of hemophilia B includes the prophylactic administration of FIX replacement therapies (i.e., plasma-derived or recombinant coagulation factor products).[1] Despite the known benefits of prophylaxis, suboptimal adherence is an issue[1,2] due to the burden of frequent intravenous infusions[2–6] and issues surrounding venous access.[2,6] Single-dose gene therapies that provide long-term expression of the deficient FIX protein would reduce treatment burden and improve quality of life for patients with hemophilia B.[1,7]

Fidanacogene elaparvovec (PF-06838435), a nonreplicating, recombinant, adeno-associated virus (AAV) vector encoding a high-activity variant of human FIX protein (FIX-R338L), is in development for the treatment of patients with moderately severe to severe hemophilia B (≤2% FIX activity).[8] FIX-R338L is differentiated from wild-type FIX by replacement of arginine with leucine at position 338 of the primary amino acid sequence,[9] which confers approximately eightfold increased activity versus wild-type FIX.[9] In a phase 1/2 study, sustained therapeutic expression of FIX-R338L activity was observed after infusion of fidanacogene elaparvovec in 15 participants with moderately severe to severe hemophilia B.[10,11] Participants were able to stop prophylaxis with minimal bleeding and factor use.[10] In a long-term follow-up study of up to 5 years, fidanacogene elaparvovec remained well-tolerated.[11]

Accurate assays to measure FIX activity are key for diagnosis, treatment, and monitoring of patients with hemophilia B.[12] One-stage clotting (OS) assays (activated partial thromboplastin time [aPTT]-based) measure the capacity of a plasma sample to reduce the prolonged coagulation time of FIX-deficient plasma calibrated against an international wild-type FIX plasma standard.[13,14] Chromogenic substrate (CS) assays are composed of purified factor concentrations and assay components, and are also calibrated with an international plasma standard.[14] However, CS assays are not approved by the U.S. Food and Drug Administration (FDA) for measuring FIX activity and are used less often than OS assays (~11% of clinical laboratories).[15] Variability in the range of FIX activity has been reported between OS assays conducted with different reagents as well as between OS and CS assays conducted at different testing sites.[12,13,16–18] Additionally, ongoing clinical trials using the high-activity FIX variant show a range of measured FIX-R338L activity between different assays.[8,13,18,19]

Differences between the wild-type factor plasma protein used as an assay standard and the endogenously expressed transgene protein may hinder interpretation of factor activity levels. Therefore, the World Federation of Hemophilia

advises more research is necessary to determine the relative accuracy of OS and CS assays in recipients of gene transfer,[1] and the FDA recommends the use of field studies with patient plasma to assess factor activity assay discrepancies.[20]

In this study, we evaluated the activity of the FIX-R338L variant in participant plasma samples from a phase 1/2a gene therapy trial of fidanacogene elaparvovec. The variation in FIX-R338L activity compared with wild-type FIX was assessed in fidanacogene elaparvovec–treated participant plasma under different assay conditions using several approaches using OS and CS assays in a global, multisite field study. Subsequently, we used the participant plasma samples to assess whether activated FIX (FIXa) contributes to measured FIX activity levels and whether the measured activity of exogenously added (spiked-in) FIX replacement products is impacted by endogenously expressed FIX-R338L.

Methods

Plasma Samples

Plasma samples were obtained from blood samples collected and processed in a phase 1/2a gene therapy trial of fidanacogene elaparvovec (NCT02484092). The trial was approved by the appropriate institutional review boards and informed consent was obtained from all individuals. Participant plasma was chosen to ensure that any translational modifications made in human liver expressing the gene therapy product were maintained, and to avoid any bias that could be introduced if purified recombinant FIX-R338L protein was used. Samples from individual participants were pooled to ensure sufficient volume to measure FIX activity. Plasma samples from multiple participants were not pooled. Briefly, previously frozen and stored plasma samples from selected participants at multiple time points >12 weeks posttreatment and not within 1 week of exogenous FIX treatment were thawed at 37°C, combined into a single tube, gently mixed, aliquoted on ice, flash-frozen, and stored at –70°C until shipping. In addition, a sufficient volume of a single nonpooled sample from two participants was available for assessing FIXa activity. Wild-type controls were generated from commercially available pooled normal human plasma samples at an initial concentration of 1.28 IU/mL (George King Bio-Medical Inc., Overland Park, Kansas, United States) diluted into FIX-deficient plasma (George King Bio-Medical) to provide nominal wild-type FIX activity levels of 0.4 IU/mL (normal) and 0.05 IU/mL (low). FIX-deficient plasma (George King Bio-Medical) was used as a negative control.

Analysis of Variability in Determining FIX-R338L Activity Levels in Participant Plasma Samples in a Multisite Field Study

An international, multisite field study was conducted to assess the activity of FIX-R338L and wild-type FIX in fidanacogene elaparvovec–treated participant plasma samples and normal control plasma samples, respectively, by both OS and CS assays in one central analytical laboratory (LabCorp [Laboratory Corporation of America], Englewood, Colorado, United States) and 18 local laboratories in 13 countries.

Four participant plasma samples were used to capture FIX-R338L activity levels ranging from normal to mild and moderate hemophilia: ≥0.4 IU/mL (high responder, designated normal), 0.25 to 0.3 IU/mL (medium responder), 0.025 IU/mL (low 1), and 0.05 IU/mL (low 2). The two low samples were generated by diluting a participant plasma sample expressing FIX-R338L levels at 0.1 to 0.15 IU/mL in FIX-deficient plasma. Two normal wild-type FIX plasma samples were included as controls. Samples were barcoded, anonymized, and sent to the local clinical laboratories and the central laboratory for analysis.

FIX activity levels were measured using validated protocols, standard reagents, and instrumentation routinely used at the respective sites (►**Supplementary Tables S1** and **S2**, available in the online version). The central laboratory used two validated OS assays using aPTT reagents (►**Supplementary Table S1**, available in the online version), Actin FSL (ellagic acid activator; BCS XP instrument; Siemens Healthcare Diagnostics Inc, Tarrytown, New York, United States) and SynthASil (silica activator; ACL TOP 700; Werfen, Orangeburg, New York, United States), and one validated CS assay, Rox Factor IX (Rox FIX; Rossix AB; ROX FIX reagent; BCS XP instrument) (►**Supplementary Table S2**, available in the online version). All 18 local laboratories performed OS aPTT assays (►**Supplementary Table S1**, available in the online version) and four also performed FIX CS assays (►**Supplementary Table S2**, available in the online version).

Central and local laboratories sent values for FIX activity to the Pfizer Clinical Research Unit, where the data were entered into an electronic health record database (IDBS E-Workbook, Woking, United Kingdom). Table values for FIX activity and geometric mean were rounded. Ratios of FIX activity geometric mean and 95% confidence intervals were determined to compare FIX activity values measured in OS and CS assays and by central and local laboratories. Assay studies have considered a difference of up to 30% in the measured level from the calculated factor treatment level, based on the labeled potency, to be clinically acceptable.[21–23] The UK Hemophilia Center Doctors' Organization guideline recommends using assays that have been studied at low, medium, and high levels and provide results within 20% of target based on potency label in samples with >30 IU/dL activity, and within 30% for samples with 10 to 30 IU/dL.[24] Therefore, in the studies reported here, activity values within 30% across assays (corresponding to 70–130% recovery) were deemed comparable.[21]

Analysis of FIXa Activity Levels in Participant Plasma Samples

FIX circulates as single-chain zymogen and, during activation, the activation peptide is removed to form FIXa before it is cleared rapidly from the blood.[25] FIXa has also been reported to be present in FIX replacement products.[25–28] Due to the properties of the assays to detect FIX, the presence of FIXa in plasma could result in overestimation of the FIX levels. Therefore, the contribution of FIXa to FIX activity levels measured in participant plasma samples was assessed.

Five plasma samples from three participants were analyzed (three pooled, two nonpooled; FIX-R338L activity ranged from 0.35–0.4 to ~0.1 IU/mL). FIXa-positive control samples were prepared by spiking commercially available purified FIXa protein (Haematologic Technologies Inc., Burlington, Vermont, United States) (ranging 0.78–50 mIU/mL) into pooled normal plasma to determine if FIXa activity was measurable in the background of wild-type FIX. Plasma controls included a FIX-deficient plasma sample, two pooled normal wild-type FIX plasma samples at 1.22 and 0.12 IU/mL each (diluted in FIX-deficient plasma), and a FIX-R338L control prepared by spiking recombinant human FIX-R338L protein (see Supplementary Methods) into FIX-deficient plasma to achieve 0.4 IU/mL FIX-R338L activity. FIXa activity was measured using a chromogenic FIXa assay (Diapharma/Rossix, ROX FIX-A) (Supplementary Methods).

Analysis of FIX Activity Following Addition of Exogenous FIX to Participant Plasma Samples

Individuals treated with fidanacogene elaparvovec may require infusion of exogenous recombinant or plasma-derived FIX products to treat bleed events or as prophylaxis before a surgical event. Therefore, exogenous replacement FIX products were spiked into participant plasma samples to determine whether endogenously expressed FIX-R338L provided by fidanacogene elaparvovec altered the determination of FIX activity. Participant plasma samples (FIX-R338L activity ranged 0.35–0.4 to ~0.1 IU/mL), a normal wild-type FIX plasma sample (0.4 IU/mL), and a FIX-deficient plasma sample (<0.01 IU/mL) were analyzed. Exogenous recombinant standard half-life (nonacog alfa), recombinant extended half-life (EHL; eftrenonacog alfa), or plasma-derived FIX protein product or buffer (as a control to represent the baseline value in each sample) were spiked into each plasma sample to reach a target therapeutic concentration of 0.50 IU/mL. The central analytical laboratory tested for FIX activity using validated OS (Actin FSL, SynthASil) and CS (ROX FIX) assays. Plasma samples were analyzed in triplicate for each of the assays, and FIX activity values were reported as mean ± standard deviation. Ratios of FIX activity geometric mean were determined to compare FIX activity values measured in OS and CS assays.

Results

Variability in FIX-R338L Activity Levels in Participant Plasma Samples in a Multisite Field Study

The aim of gene therapy with fidanacogene elaparvovec expressing the FIX-R338L variant is to achieve sustained therapeutic FIX activity levels. It is therefore important to understand the range of FIX-R338L activity results across different assays and different laboratories.

Enrollment of a central laboratory and 18 local laboratories enabled the evaluation of inter-laboratory variability for the OS assays. The central laboratory used the validated ellagic acid–based Actin FSL and silica-based SynthASil OS assays. Across the local laboratories that performed OS assays, eight different OS reagents were used: ellagic acid ($n = 6$), silica, SynthASil ($n = 5$), other silica activators ($n = 4$), kaolin ($n = 2$), and polyphenols ($n = 1$). A range of FIX activity was observed across OS assays using other aPTT activators (other silica activators, kaolin, and polyphenols) (►**Supplementary Fig. S1** and ►**Supplementary Table S3**, available in the online version). Silica-based activators (SynthASil + others) were the most predominant OS reagents used by 50% of the sites.

Comparisons of FIX activity were made across the three validated assays at the central laboratory. FIX activity values were consistently higher with the silica-based OS aPTT SynthASil assay compared with ellagic acid–based OS Actin FSL and CS Rox FIX assays. The higher activity values were more pronounced in the participant (FIX-R338L) versus control (wild-type FIX) plasma samples (►**Table 1**). There was an apparent trend to higher SynthASil to Actin FSL and SynthASil to Rox FIX ratios, with lower FIX activity values for both participant and control plasma samples. The ratios of SynthASil to Actin FSL FIX activity were 4.34 and 1.84 for the participant samples with the lowest and normal FIX-R338L activity, respectively (►**Table 1**). The ratios of SynthASil to

Table 1 Central laboratory assessment of participant and control plasma samples by OS and CS assays

	Actin FSL (OS)		SynthASil (OS)		Rox Factor IX (CS)	
Plasma sample	IU/mL	SynthASil/Actin FSL ratio	IU/mL	SynthASil/SynthASil ratio	IU/mL	SynthASil/Rox FIX ratio
Participant sample (FIX-R338L)						
Low 1 (0.025 IU/mL)	0.013	4.34	0.057	1	0.014	3.98
Low 2 (0.05 IU/mL)	0.025	3.69	0.094	1	0.026	3.56
Medium (0.25–0.3 IU/mL)	0.178	2.27	0.405	1	0.186	2.18
Normal (≥0.4 IU/mL)	0.404	1.84	0.743	1	0.363	2.05
Control sample (wild-type FIX)						
Low (0.05 IU/mL)	0.047	1.79	0.084	1	0.046	1.81
Normal (≥0.4 IU/mL)	0.390	1.16	0.451	1	0.370	1.22

Abbreviations: CS, chromogenic substrate; FIX, factor IX; OS, one-stage.
Note: Assays were performed in a single central laboratory ($N = 1$).

Actin FSL FIX activity were 1.79 and 1.16 for the control plasma samples with low and normal wild-type FIX activity, respectively (►**Table 1**). A similar pattern was observed for the ratio of SynthASil to Rox FIX activity, specifically a trend to higher ratios with lower FIX activity values. In contrast to the OS SynthASil assay, FIX activity using the OS Actin FSL assay was closely aligned with the CS Rox FIX assay (►**Table 1**). Similar results were also seen when comparing FIX activity in the local laboratory SynthASil assays versus the central laboratory Actin FSL assay (►**Table 2**).

In general, FIX-R338L activity in the patient samples was higher when measured via the SynthASil assay at the central laboratory versus local laboratory, albeit within ± 30% (►**Table 2**). The ratios of local SynthASil to central SynthASil laboratory FIX activity ranged between 0.72 and 0.89 for participant samples (FIX-R338L) and 0.71 and 0.88 for plasma control samples (wild-type FIX) (►**Table 2**). The ratios of FIX activity measured in the wild-type FIX plasma control samples ranged between 0.71 (local SynthASil to central SynthASil) and 1.27 (local SynthASil to central Actin FSL) (►**Table 2**).

Four laboratories performed CS assays (Biophen FIX, $n = 2$; Rox FIX, $n = 2$). Both CS assay kits contained human source reagents, synthetic phospholipids, and different chromogenic factor Xa substrates. Similar FIX activity values were observed in participant FIX-R338L and wild-type FIX control samples with the CS assay across local and central laboratories, with ratios of local to central laboratory FIX activity ranging from 0.91 to 1.16 (►**Table 3**). The FIX activity values in both participant and plasma control samples determined using the Actin FSL OS assay were closely aligned to the CS assays, with ratios of local laboratory CS to central laboratory Actin FSL ranging from 0.90 to 1.27 (►**Table 3**).

Analysis of FIXa Activity Levels in Participant Plasma Samples

FIX activity in plasma samples was determined using an OS assay prior to assessing whether FIXa was present in the plasma sample. FIX activity ranged from 0.107 to 0.457 IU/mL in participant plasma samples and was 0.441 and 0.227 IU/mL in the wild-type control plasma samples (►**Supplementary Table S4**, available in the online version).

The FIXa chromogenic was highly sensitive for FIXa activity, detecting FIXa activity in the FIXa-positive controls ranging from as low as 0.9 to 38.3 mIU/mL (►**Table 4**). Despite this level of sensitivity, no FIXa activity was detected in the participant plasma samples, the wild-type plasma controls, the FIX-deficient plasma sample, or the recombinant human FIX-R338L protein-spiked FIX-deficient plasma sample (►**Table 4**).

Analysis of FIX Activity Following Addition of Exogenous FIX to Participant Plasma Samples

FIX activity values with exogenous recombinant standard half-life, recombinant EHL, and plasma-derived FIX protein products spiked into FIX-deficient, participant and normal pooled plasma samples were evaluated in two OS assays, SynthASil and Actin FSL, and the Rox factor IX CS assay

Table 2 Central laboratory and local laboratory assessment of participant and control plasma samples by OS assays

Plasma sample	Geometric mean, IU/mL				Ratio (95% CI)			
	Local SynthASil (OS)	Central SynthASil (OS)	Local Actin FSL (OS)	Central Actin FSL (OS)	Local Actin FS (OS)	Local SynthASil (OS)/Central SynthASil (OS)	Local SynthASil (OS)/Central Actin FSL (OS)	Local Actin FS (OS)/Central Actin FSL (OS)	Local Actin FSL (OS)/Central Actin FSL (OS)
Participant sample (FIX-R338L)									
Low 1 (0.025 IU/mL)	0.041	0.057	0.012	0.013	0.036	0.72 (0.39–1.32)	3.11 (1.69–5.73)	2.77 (0.28–27.26)	0.92 (0.00–1,406,853)
Low 2 (0.05 IU/mL)	0.067	0.094	0.027	0.025	0.054	0.71 (0.43–1.17)	2.63 (1.60–4.31)	2.14 (1.33–3.42)	1.05 (0.00–3,876.37)
Medium (0.25–0.3 IU/mL)	0.360	0.405	0.241	0.178	0.330	0.89 (0.74–1.07)	2.02 (1.68–2.44)	1.85 (1.18–2.92)	1.35 (1.10–1.67)
Normal (≥0.4 IU/mL)	0.661	0.743	0.428	0.404	0.653	0.89 (0.67–1.18)	1.64 (1.23–2.18)	1.62 (0.93–2.83)	1.06 (0.16–7.00)
Control sample (wild-type FIX)									
Low (0.05 IU/mL)	0.059	0.084	0.037	0.047	0.049	0.71 (0.38–1.30)	1.27 (0.69–2.34)	1.05 (0.87–1.25)	0.79 (0.05–12.58)
Normal (≥0.4 IU/mL)	0.399	0.451	0.408	0.390	0.382	0.88 (0.72–1.08)	1.02 (0.83–1.26)	0.98 (0.93–2.83)	1.05 (0.94–1.17)

Abbreviations: CI, confidence interval; FIX, factor IX; OS, one-stage.
Note: SynthASil assays were conducted in four or five local laboratories ($N = 4$–5) and one central laboratory ($N = 1$); Actin FSL assays were conducted in two local laboratories ($N = 2$) and one central laboratory ($N = 1$); Actin FS assays were conducted in three local laboratories ($N = 3$).

Table 3 Comparison of central laboratory and local laboratory assessment of participant and control plasma samples by CS assay

Plasma Sample	Geometric mean, IU/mL			Ratio (95% CI)	
	Local Lab CS	Central Lab CS	Central Lab Actin FSL OS	Local Lab CS/Central Lab CS	Local Lab CS/Central Lab Actin FSL OS
Participant sample (FIX-R338L)					
Low 1 (0.025 IU/mL)	0.017	0.014	0.013	1.16 (0.26–5.08)	1.27 (0.29–5.55)
Low 2 (0.05 IU/mL)	0.025	0.026	0.025	0.94 (0.10–8.60)	0.98 (0.11–8.92)
Medium (0.25–0.3 IU/mL)	0.172	0.186	0.178	0.93 (0.39–2.22)	0.97 (0.40–2.31)
Normal (≥0.4 IU/mL)	0.421	0.363	0.404	1.16 (0.87–1.54)	1.04 (0.78–1.39)
Control sample (wild-type FIX)					
Low (0.05 IU/mL)	0.042	0.046	0.047	0.91 (0.24–3.49)	0.90 (0.23–3.47)
Normal (≥0.4 IU/mL)	0.394	0.370	0.390	1.06 (0.62–1.82)	1.01 (0.60–1.72)

Abbreviations: CI, confidence interval; CS, chromogenic substrate; FIX, factor IX; Lab, laboratory; OS, one-stage.
Note: The CS assays were conducted in four local laboratories ($N = 4$) and one central laboratory ($N = 1$).

Table 4 Assessment of FIXa activity in participant and control plasma samples using a FIXa chromogenic assay

Plasma sample	mIU/mL[a]
Assay control	
FIXa assay control (range 0.41–0.61 mIU/mL)	0.6
Positive control (FIXa protein spiked into pooled normal wild-type FIX plasma)	
FIXa at 50 mIU/mL	38.3
FIXa at 25 mIU/mL	15.6
FIXa at 12.5 mIU/mL	11.2
FIXa at 6.25 mIU/mL	5.6
FIXa at 3.125 mIU/mL	2.8
FIXa at 1.56 mIU/mL	1.4
FIXa at 0.78 mIU/mL	0.9
Plasma control	
Pooled normal wild-type FIX plasma (FIX 1.22 IU/mL)	ND
Pooled normal wild-type FIX plasma (FIX 0.12 IU/mL)	ND
FIX-deficient plasma	ND
FIX-R338L protein (0.4 IU/mL) spiked into FIX-deficient plasma	ND
Individual participant plasma	
Pooled participant plasma 1	ND
Pooled participant plasma 2	ND
Pooled participant plasma 3	ND
Non-pooled participant plasma 1	ND
Non-pooled participant plasma 3	ND

Abbreviations: FIX, factor IX; FIXa, activated factor IX; ND, not detected.
Note: The FIXa calibrator and control were standardized to international standards.
[a]1,000 mIU/mL = 1 IU/mL.

(►**Fig. 1**). The addition of exogenous replacement FIX product to FIX-deficient plasma resulted in the expected FIX activity values in all three assays (►**Fig. 1A**). The addition of FIX replacement products to reach a target therapeutic concentration of 0.5 IU/mL was additive to the FIX activity values measured in the buffer-spiked baseline participant and plasma control samples alone, resulting in the expected FIX activity values in all three assays (►**Fig. 1B–E**). In the three individual participant buffer-spiked plasma samples, FIX activity values measured using the SynthASil assay were generally higher than with the Actin FSL assay (►**Fig. 1B–D**). The SynthASil to Actin FSL ratio ranged from 1.40 to 2.22 for the three buffer-spiked participant samples (►**Supplementary Table S5**, available in the online version).

Discussion

FIX-R338L, the high-activity variant of human FIX protein, is emerging as the FIX protein of choice for use in AAV-directed gene therapy for the treatment of patients with hemophilia B.[10,29,30] Efficacy and safety data from phase 1/2 and 3 studies are encouraging[7,10,11,29–31] and suggest the potential of gene therapy using this high-activity variant as a treatment option for patients with moderately severe to severe hemophilia B. Indeed, an AAV-based gene therapy delivery FIX-R338L was recently approved by the FDA.[32] However, it is important to develop a greater understanding of the impact of this variant on currently available laboratory assays to assess FIX activity. There is growing evidence that different OS and CS assays to assess FIX activity may produce a range of results, with some exceeding generally accepted margins of variation.[8,18] Further, mechanistic data to explain the root cause of the range of values observed are limited.[18] In addition, as exogenous replacement wild-type FIX products may be needed to manage bleeding episodes in patients treated with gene therapy, it is important to understand the impact of basal FIX-R338L on the determination of FIX activity. Collectively, this information is needed to optimize

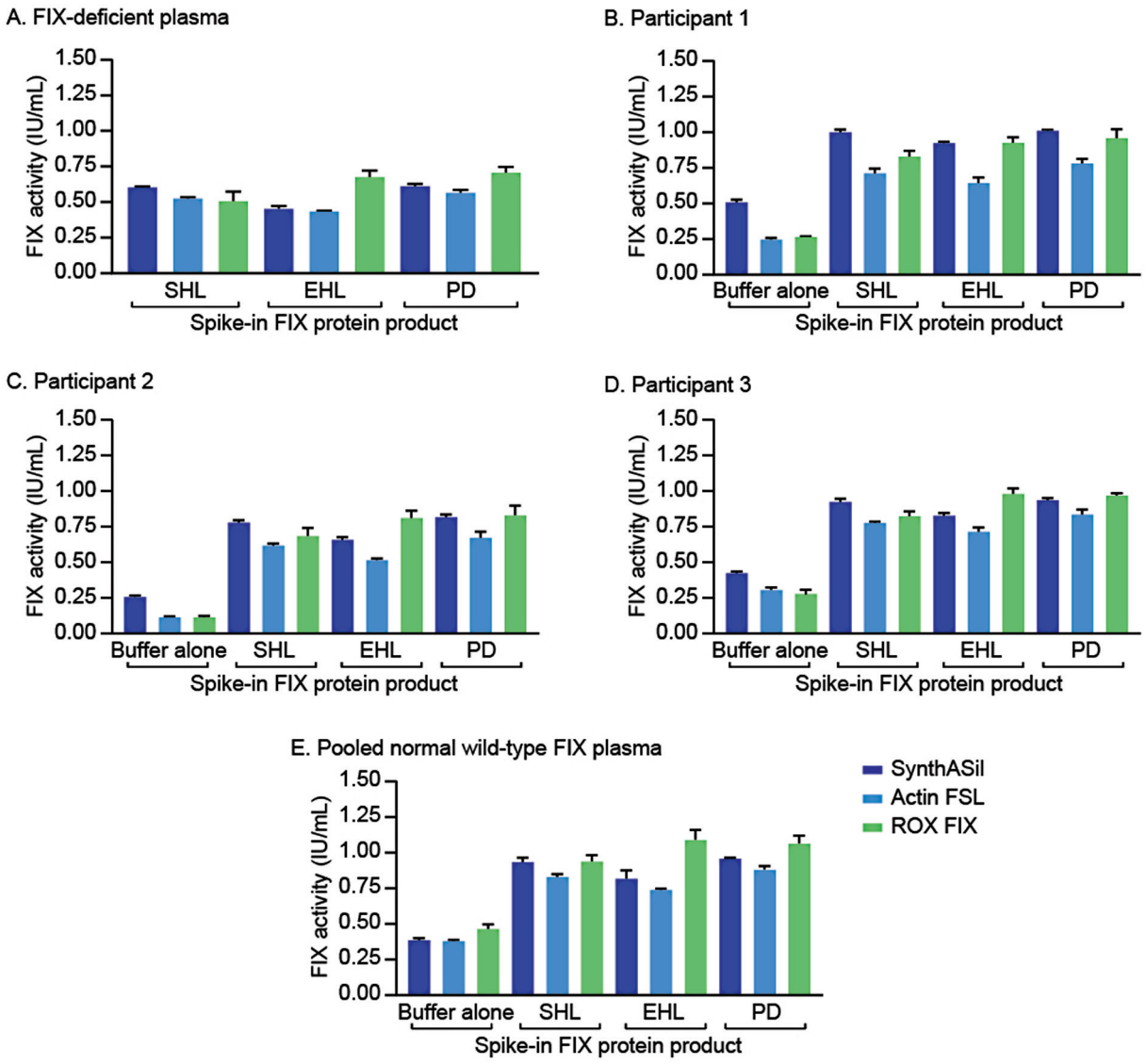

Fig. 1 Measurement of FIX activity following addition of exogenous FIX to plasma samples from fidanacogene elaparvovec–treated patients. Exogenous FIX (SHL, nonacog alfa; EHL, eftrenonacog alfa; plasma-derived) was spiked into FIX-deficient plasma, participant plasma (participants 1–3), and pooled normal wild-type plasma to reach a target therapeutic concentration of 0.50 IU/mL. Buffer was added to the participant plasmas or pooled normal plasma as a control to measure the baseline values. Plasma samples were analyzed in triplicate, and FIX activity values are shown as mean ± standard deviation IU/mL. CS, chromogenic substrate; EHL, extended half-life; FIX, factor IX; OS, one-stage; PD, plasma-derived; SHL, standard half-life.

the management of patients with hemophilia B following AAV-directed gene therapy using the FIX-R338L variant. The analyses reported here aim to help address these uncertainties.

The results from this field study support previous findings that a range of FIX activities may be determined for a given sample depending on the specific assay and/or reagents used.[8,18] In the study described here, we used fidanacogene elaparvovec–treated participant plasma with endogenously expressed FIX-R338L from a phase 1/2 gene therapy trial (NCT02484092) sent to 18 different local laboratories as well as the central laboratory. Overall, we found FIX-R338L activity values were higher in the OS silica-based SynthASil assay compared with the OS ellagic acid–based Actin FSL assay. This trend was also observed in the control samples with wild-type FIX, although the range in FIX activity was smaller than with FIX-R338L. The variation in activity was most notable at the lowest FIX activity levels. Despite this, results were generally consistent across the central and local laboratories, suggesting the variation lies within the reagent activation stage rather than a user variation. Studies suggest the range of FIX activity levels observed between assays could be linked to the aPTT reagents, activating reagent, and phospholipid content used to measure FIX activity and also the dynamics of factor VIII (FVIII) activation.[18,33] Eight different aPTT reagents were used in the OS assays across the laboratories participating in this field study, with inter-laboratory variability observed across the different OS reagents used. Also, in the assays described here, activity was calibrated to a validated wild-type FIX plasma standard. The similarities in FIX activity levels of endogenously expressed, transgene-derived FIX-R338L, recombinant

FIX-R338L spiked into patient plasma samples, and from plasma samples derived from the original family where the R338L mutation was first described across OS and CS assays, indicate that differences between assay results are likely attributable to the protein itself as opposed to any modifications specific to FIX-R338L transgene expression.[8,9,18]

The choice of plasma-derived, recombinant, or EHL wild-type products, or the high-activity FIX-R338L variant also influences the range of FIX activity measured.[8,17,18,34] For example, OS assays overestimated FIX activity of N-glyco-PEGylated recombinant FIX (nonacog beta pegol, N9-GP)[35] but underestimated the potency of a recombinant FIX-albumin fusion protein.[36] Correlation with bleeding events and FIX activity by assay may therefore be helpful in interrogating the significance of the range of FIX activities produced by the different assays. Regardless, these data suggest the type of assay used may be important when assessing the FIX activity for patients after gene therapy with the FIX-R338L variant.

Although only four local laboratories measured activity using two different CS assays, the geometric mean ratios for FIX activity of both FIX-R338L and wild-type FIX were similar between the central and local laboratory assessments. In a field study where purified human recombinant FIX-R338L protein was spiked into pooled inhibitor-free congenital hemophilia B patient plasma achieving nominal FIX activities ranging from 0.05 to 1.5 IU/mL based on the values measured using the OS SynthASil FIX assay, assay variability was also observed across 38 laboratories in 11 countries, with a threefold variation between OS and CS assays.[18] Although the study reported here used study participant plasmas and not recombinant protein-spiked samples, the results are consistent with those observed in this study in that particulate activators (e.g., silica, kaolin) resulted in higher FIX-R338L activities than ellagic acid, and as FIX activity decreased, assay variability increased.[18] In both cases, the differences in FIX-R338L activity between the OS SynthASil assay and the Rox CS assay were similar.[18]

The presence of residual FIXa in plasma from participants treated with fidanacogene elaparvovec could explain the increased FIX-R338L activity observed relative to wild-type FIX protein.[9] FIXa was not detected in the plasma from these participants using a FIXa CS assay. These data therefore support that the increased activity seen with FIX-R338L is not due to increased/residual FIXa in the plasma samples. Additionally, this suggests the range of FIX activities observed across different assays is not driven by residual FIXa.

Due to the complexity and the propriety nature of the OS reagents, it is difficult to determine the exact mechanism contributing to assay differences. Additionally, the assays used in local laboratories used different protocols, plasma standards, and instruments. OS assays are kinetic assays that measure time to formation of a fibrin clot, and the composition of the OS reagent may impact the formation of the tenase complex, potentially amplifying the difference between wild-type FIX and FIX-R338L. In a study with a pairwise comparison of similarly purified wild-type FIX or FIX-R338L proteins spiked at equimolar protein concentrations into hemophilia B plasma, a range in the absolute clotting times was observed between the Actin FS, Actin FSL, and SynthASil OS reagents.[37] The differences in the absolute clotting time between the wild-type FIX and FIX-R338L were more pronounced at lower concentrations in the three different OS assay reagents, with greater differences observed between FIX-R338L and wild-type FIX in SynthASil. One possible mechanistic explanation for the discordance between different FIX assays in measuring FIX-R338L may be through factor XI activation (FXIa). Studies have demonstrated that small amounts of activated FVIII (FVIIIa) are generated during the pre-incubation step in the OS assay before the addition of calcium, through the action of FXIa.[38] In a study assessing FXIa activity at short-time intervals pre-calcification, the ellagic acid–based Actin FS and Actin FSL generated more FXIa compared with the SynthASil (silica) reagent at all time points.[37] This is also consistent with a study using purified reagents, in which more FXIa was generated in OS ellagic acid–based reagents compared with the OS silica-based reagents.[18] Mechanistic studies with the recombinant FIX-R338L variant demonstrated that the enhanced activity of FIX-R338L is dependent on its interaction with FVIIIa and FX on phospholipid surfaces in the tenase complex.[39] Thus, minor differences in the FVIIIa generation in different aPTT reagents may be amplified in the reaction after calcium addition.

The aim of gene therapy using the high-activity FIX variant is for patients with moderately severe to severe hemophilia B to endogenously express FIX-R338L at levels that result in clinical benefit. However, there may be times when an infusion of exogenous replacement FIX is required for a breakthrough bleed and/or for prophylaxis prior to a surgical event. To ensure effective management of patients who have received gene therapy with the FIX-R338L variant, it is important to determine whether the combination of exogenous FIX products and endogenous FIX-R338L are additive. The addition of exogenous replacement FIX products at a therapeutic concentration of 0.5 IU/mL to the fidanacogene elaparvovec participant plasma or pooled normal wild-type FIX samples was additive, showing that established correction calculations can be leveraged for recipients of FIX-R338L gene therapy who need replacement FIX infusion.

With the prospect of gene therapy for patients with hemophilia B, the assessment of FIX activity following viral vector administration is of interest for the assessment of therapeutic efficacy. There is no international standard for FIX-R338L activity measurement, so it is important to understand and quantify variability in available FIX activity assays. However, given the differences in measurement of factor activity between assays, correlation with clinical response is important for determination of FIX-R338L activity in recipients of gene therapy.[1]

In conclusion, we have demonstrated a range of FIX activity across OS and CS assays in fidanacogene elaparvovec–treated participant plasma samples from a phase 1/2a gene therapy trial. These findings are supportive of prior data

and add further insight that the observed range is more sensitive at lower FIX activity levels. Emerging data from multiple ongoing clinical studies will provide some understanding of the overall clinical impact of these findings. Clinicians assessing patients who have received gene therapy with the FIX-R338L variant may find it helpful to familiarize themselves with the different OS and CS assays available to assess FIX activity and contextualize the results for patient management.

What is known about this topic?

- Fidanacogene elaparvovec, a hemophilia B gene therapy, delivers the high-activity factor IX variant FIX-R338L.
- Variability in the range of factor IX activity has been reported between one-stage (OS) assays conducted with different reagents as well as between OS and chromogenic substrate (CS) assays conducted at different testing sites monitoring factor IX activity.
- Emerging data from ongoing clinical trials using the high-activity factor IX variant suggest a range in measured FIX-R338L activity between different OS and CS assays.
- Understanding the activity of FIX-R338L in clinical samples is critical.

What does this paper add?

- FIX activity was measured using OS and/or chromogenic assays by the central and local laboratories.
- FIX-R338L activity was higher with OS silica–based versus OS ellagic acid–based and chromogenic assays.
- These findings provide insight into the variability between assays when measuring FIX with endogenously produced FIX-R338L and will help establish best practices for measuring FIX-R338L activity in patients undergoing fidanacogene elaparvovec gene therapy.

Data Availability Statement

Upon request, and subject to review, Pfizer will provide the data that support the findings of this study. Subject to certain criteria, conditions, and exceptions, Pfizer may also provide access to the related individual de-identified participant data. See https://www.pfizer.com/science/clinical-trials/trial-data-and-results for more information.

Authors' Contribution

All authors had full access to the data, and all authors contributed to data interpretation and the drafting, critical review, and revision of the manuscript. All authors granted approval of the final manuscript for submission.

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Conflict of Interest

C.C., J.M., C.Y.T., J.Z.L., P.G., and J.R. are employees of Pfizer and may own stock/options in the company. D.D.P., H.S., S.R., J.-C.M., and J.E.M. were employees of Pfizer at the time of the study conduct and may own stock/options in the company.

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