Received: 24 August 2020 | Accepted: 22 February 2021

DOI: 10.1111/jth.15281

ORIGINAL ARTICLE

Factor IX assay discrepancies in the setting of liver gene therapy using a hyperfunctional variant factor IX-Padua

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Funding information
This study was supported by Spark Therapeutics and Pfizer Inc. B.J.S.-J. is supported by a grant from the National Institutes of Health/National Heart, Lung, and Blood Institute (1K08HL140078). L.A.G. was supported by a grant from the National Institutes of Health/National Heart, Lung and Blood Institute (K08 HL 146991-01). V.R.A. was supported by a grant from the National Institutes of Health/National Heart, Lung, and Blood Institute (PO1 HL 139420, Project 4).

Abstract

Background: Limited information exists regarding the factor IX (FIX) coagulant activity (FIX:C) measured by different assays following FIX-Padua gene therapy.

Objective: Assess for the first time FIX:C in five commonly used coagulation assays in plasma samples from hemophilia B subjects receiving FIX-Padua gene transfer.

Methods: FIX:C was compared between central (n = 1) and local laboratories (n = 5) in the study, and across four commonly used FIX:C one-stage assays and one FIX:C chromogenic assay. For comparison, samples of pooled congenital FIX-deficient plasma spiked with purified recombinant human FIX (rHFIX)-Padua protein or rHFIX (nonacog alfa) to obtain FIX:C concentrations from ~20% to ~40% were tested.

Results: FIX:C results at local laboratories strongly correlated with central laboratory results. However, absolute values at the central laboratory were consistently lower than those at local laboratories. Across five different FIX:C assays, a consistent pattern of FIX:C was observed for subjects receiving fidanacogene elaparvovec-expressed gene transfer. Use of Actin FSL activated partial thromboplastin time (APTT) reagent in the central laboratory resulted in lower FIX:C values compared with other APTT reagents tested. The chromogenic assay determined lower FIX:C than any of the one-stage assays. The rHFIX-Padua protein–spiked samples showed similar results. In contrast, FIX:C results for rHFIX-nonacog alfa measured within 25% of expected for all one-stage assays and below 25% in the chromogenic assay.

Conclusions: Assay-based differences in FIX:C were observed for fidanacogene elaparvovec transgene product and rHFIX-Padua protein, suggesting the variable FIX:C determined with different assay reagents is inherent to the FIX-Padua protein and is not specific to gene therapy–derived FIX-Padua.

KEYWORDS
blood coagulation tests, biological assay, fidanacogene elaparvovec, genetic therapy, hemophilia B

Manuscript Handled by: Robert Gosselin, 22 February 2021

Essentials

- Understanding FIX coagulant activity (FIX:C) assay discrepancies during gene therapy is critical.
- Assay-dependent differences in FIX:C were observed across the 5 commonly used assays tested.
- Although determined FIX:C values varied between assays, there was a correlation between assays.
- Assay selection may be important when measuring FIX:C following FIX-Padua gene transfer.

1 | INTRODUCTION

Gene therapy for hemophilia B is progressing through clinical trials, with a number of active candidates in clinical development.[1] Fidanacogene elaparvovec (formerly PF-06838435, SPK-9001) is an adeno-associated virus–based vector designed for hepatocyte-specific expression of factor IX Padua (FIX-R338L) and is currently in phase 3 of clinical development for the treatment of hemophilia B (NCT03861273).[1-4] Preliminary data from a phase 1/2a study in 15 adult men with hemophilia B (FIX coagulant activity [FIX:C] ≤2%) who received fidanacogene elaparvovec at a dose of 5×10^{11} vg/kg showed that subjects, on average, achieved sustained, steady-state FIX activity of 33.7% as measured by one-stage clotting assays in local laboratories at treatment sites.[2] The annualized bleeding rate in participants significantly decreased from a mean of 11.1 (range, 0-48) events per year before vector administration to a mean of 0.4 (range, 0-4) events per year after administration ($p = .02$).[2] No serious adverse events were reported during or after fidanacogene elaparvovec infusion.

The one-stage clotting assay, based on the activated partial thromboplastin time (APTT), is the routine method used to evaluate FIX:C to monitor individuals with hemophilia B. A wide variety of reagents and assay platforms are in clinical use for one-stage clotting assays, leaving considerable potential for variability in determined FIX:C. This variability has been shown when measuring novel recombinant FIX products[5-7] and, in fact, has been observed when comparing local and central laboratory determinations of FIX:C in plasma from subjects treated with fidanacogene elaparvovec in the phase 1b/2 study. This variability may, in part, result from the biochemical basis of FIX-Padua's gain of function hemostatic effect. An orthogonal two-stage chromogenic assay is also in limited use in the United States (not Food and Drug Administration approved) and Europe, with only two reagents available (Hyphen BioMed and Rossix). The objective of the current study was to assess the FIX:C of the fidanacogene elaparvovec–expressed transgene product in plasma samples collected from subjects in the phase 1/2a study[2] via comparison for the first time of FIX:C between central and local laboratories and as determined by four commonly used FIX:C aPTT reagents and one FIX:C chromogenic assay.

2 | METHODS

Details regarding the methodology for the phase 1/2a study, which was conducted in North America and Australia, were previously reported.[2] Plasma samples collected from subjects treated with fidanacogene elaparvovec were tested centrally (Colorado Coagulation, Laboratory Corporation of America, Englewood, CO) and locally at the site where subjects were being followed using the FIX:C assay routinely used by the clinical laboratory at the subject's clinical trial enrollment site. All laboratories, local and central, used one-stage clotting assays. The central laboratory used the Actin FSL reagent, an ellagic acid activator, on a BCS XP analyzer (Siemens Healthcare Diagnostics). The following assays were in use at the local laboratory sites: TriniCLOT/STA-R Evolution (TCoag, Ireland; TriniCLOT Automated APTT contains a platelet factor 3 reagent [rabbit brain phospholipids] plus a particulate activator [micronized silica] in a suitable buffer), Hemosil aPTT-SP/ACL TOP (Instrumentation Laboratory), Dade Actin FS/BCS XP analyzer (Siemens Healthcare Diagnostics), STA-PTT/STAR-R analyzer (Diagnostica Stago, Inc.), and HemosIL SynthASil, IL-Top analyzer (Instrumentation Laboratory).

Five samples collected from three subjects who received fidanacogene elaparvovec gene therapy in the phase 1/2a study were received and stored frozen at −70°C until day of testing. FIX:C was measured in four *in vitro* diagnostic (IVD) FIX:C one-stage clotting assays and in one FIX chromogenic assay (Rossix) (Table 1). The FIX APTT one-stage clotting assays selected were the most common APTT reagents used in College of American Pathologists–accredited laboratories in North America in 2017 and represent the three main types of activators commonly used in the FIX one-stage clotting assay: silica (STA-PTT Automate [Diagnostica Stago, Inc.], HemosIL SynthASil [Instrumentation Laboratory]), ellagic acid (Dade Actin FSL [Siemens Healthcare Diagnostics]), and kaolin (STA-C.K. Prest [Diagnostica Stago, Inc.]).[8] For comparison, FIX:C was also determined in pooled congenital FIX-deficient plasma (George King Bio-Medical, Inc.) spiked with purified recombinant human FIX (rHFIX)-Padua protein as described by Samelson-Jones et al[4] and rHFIX-nonacog alfa product (nonacog alfa, manufactured by Pfizer Inc., provided by Spark Therapeutics, Inc.) in each of the five FIX:C assays. Recombinant human FIX-Padua is produced in human embryonic kidney 293 cells, and rHFIX-nonacog alfa is produced in Chinese hamster ovary cells.[4,9] Based on the assumption that Padua-specific activity of rHFIX is approximately

TABLE 1 Commonly used FDA-approved FIX:C one-stage clot and chromogenic assay systems

Parameter	FIX:C APTT One-stage Clotting Assays				FIX:C Chromogenic Assay
Manufacturer	Siemens Healthcare Diagnostics	Instrumentation Laboratory	Diagnostica Stago	Diagnostica Stago	ROSSIX
Instrument	BCS XP	ACL TOP	STA-R Evolution	STA-R Evolution	BCS XP
Reagent	Actin FSL	HemosIL SynthASil	STA-C.K. Prest	STA-PTT Automate	ROX Factor IX Kit
Activator	Ellagic acid	Silica	Kaolin	Silica	NA
% CAP-accredited laboratories (2017)	23%	26%	5%	34%	NA

Abbreviations: APTT, activated partial thromboplastin time; CAP, College of American Pathologists; FDA, US Food and Drug Administration; FIX, factor IX; FIX:C, coagulant activity of factor IX; NA, not applicable.

8-fold higher than that of wild-type FIX, the rHFIX-Padua protein was prepared at 20× the protein concentrations of 5000, 3760, and 2500 ng/ml to approximate FIX:C of 40%, 30%, and 20% (assuming approximately 8-fold greater specific activity[4]) when diluted to final concentration in the one-stage clotting assay. Concentrations were determined by absorbance at 280 nm using an extinction coefficient of 1.32 mg/ml/cm. The rHFIX-nonacog alfa product was prepared at 20× the FIX:C concentrations of 800%, 600%, and 400%. Both proteins were diluted in 20 mmol/l hydroxyethyl piperazine ethane sulfonic acid, 150 mmol/l sodium chloride, 2 mmol/l calcium chloride, and 0.1% polyethylene glycol-800, then further diluted 1:20 into pooled congenital FIX-deficient plasma on the day of testing to provide samples with approximate FIX:C of 40%, 30%, and 20%.

The FIX:C assays were calibrated using calibrators with FIX activity values provided by the manufacturer in % (IU/dl), traceable to the World Health Organization's international standards. Quality control, subject, rHFIX-Padua, and rHFIX-nonacog alfa samples were tested at the central laboratory in accordance with manufacturer recommendations and/or following existing laboratory standard operating procedures. Subject samples were tested in singlicate; the rHFIX-Padua–spiked and rHFIX-nonacog alfa–spiked plasma samples were tested in triplicate. Sample testing in the respective IVD FIX one-stage clotting assays was performed at three dilutions prepared by the coagulation analyzer, and sample testing in the FIX chromogenic assay was performed at single dilution. To standardize, the reported FIX one-stage clotting result was determined using an algorithm that assessed sample linearity and parallelism to the calibration curve using at least two dilution results.

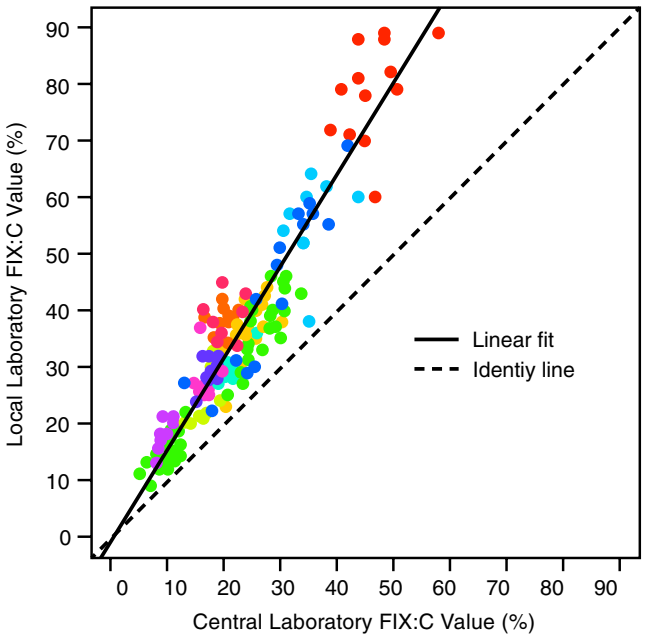

FIGURE 1 Pairwise comparison of FIX:C at local laboratories versus the central laboratory for subjects enrolled in the phase 1/2a fidanacogene elaparvovec study. Linear model of the formula FIX_local = a + b*FIX_central was fit to steady-state FIX:C data to quantify the relationship between local and central laboratory values. The linear model had the following estimates of the coefficients: a = −0.26 (95% CI, −5.86 to 5.34; p = .92); b = 1.60 (95% CI, 1.38-1.83; p < .001). Data are color-coded by individual subjects. CI, confidence interval; FIX:C, coagulant activity of factor IX

3 | RESULTS AND DISCUSSION

Steady-state FIX:C from the first 15 subjects in the study revealed a strong correlation between results obtained at local laboratory sites and those obtained at the central laboratory. However, the absolute values obtained at the central laboratory were consistently lower than those obtained at local laboratories. Results from the pairwise comparison between FIX:C as determined by local laboratory analysis versus central laboratory analysis for every sample assayed in the study are shown in Figure 1. On average, results from the local laboratories were approximately 60% higher than results measured in the central laboratory.

To gain further insight into the difference between central and local laboratory results, select subject plasma samples, representative of the range of FIX:C seen in the phase 1/2 study, were also measured in four commonly used IVD-approved FIX:C one-stage clotting assays and in a single FIX:C chromogenic assay. In these FIX:C assays, the plasma samples from subjects receiving fidanacogene elaparvovec gene therapy showed a consistent pattern of FIX:C activity in the five assays tested (Figure 2). However, results for the individual assays differed. For the APTT-based FIX:C assays, Actin FSL gave the lowest FIX:C values, whereas STA-PTT Automate gave the highest FIX:C values for most samples. The highest observed

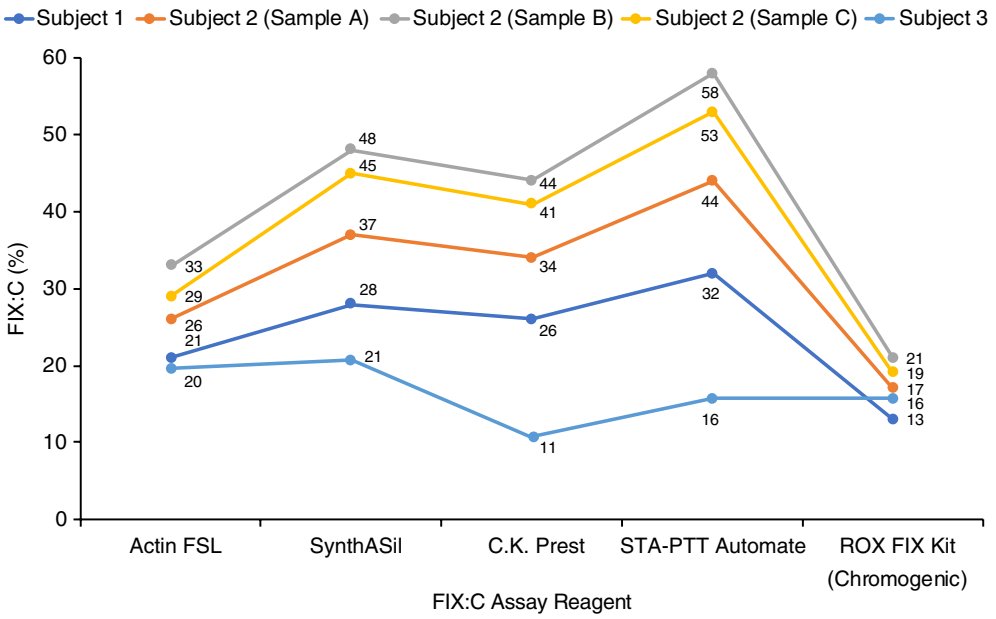

FIGURE 2 FIX:C measured in plasma from subjects who received fidanacogene elaparvovec gene therapy. FIX:C, coagulant activity of factor IX

values across all of the subjects' samples fell within a range of 1.5- to 1.9-fold higher than the lowest observed values. For all samples, the chromogenic assay gave the lowest FIX:C values; the observed FIX:C values were also less variable across subjects with the chromogenic assay than with the one-stage clotting assays.

A similar FIX:C assay reagent–dependent pattern was observed for samples spiked with purified rHFIX-Padua to approximate FIX:C of 40%, 30%, and 20% of normal (Figure 3A). Like the FIX-Padua gene therapy subject samples, the same relative differences were observed with the different reagents in the one-stage clotting assays with the recombinant FIX-Padua protein: Actin FSL produced the lowest values and STA-PTT Automate tended to produce the highest values. The chromogenic assay provided the lowest activity levels for the rHFIX-Padua–spiked samples.

In contrast to the rHFIX-Padua–spiked plasma samples, rHFIX-nonacog alfa–spiked plasma samples recovered within an acceptable range of expected concentrations (±25%) in all of the APTT-based IVD-approved FIX:C assays (Figure 3B). This finding could, in part, be due to distinct posttranslational modifications imparted to the expressed protein by distinct cell lines. Concentrations observed with the chromogenic assay were 26.0%, 20.7%, and 13.0% for the 40%, 30%, and 20% rHFIX-nonacog alfa–spiked samples, respectively. These values were lower than those observed with the one-stage clotting assays. These effects are likely magnified in the rHFIX-Padua samples because of the high specific activity of the variant protein.

Assay-based discrepancies in FIX and FVIII activity for the diagnosis of hemophilia and for the measured potency of plasma-derived factor concentrates and recombinant replacement factors are well established.[5,10-14] Differences in assay methodologies, activation time, and reagent composition (activator and phospholipid content) may result in variable measurements that complicate label standardization of potency across currently available products and gene therapy products, based on not only adeno-associated virus technology but other vector systems as well.[14,15] Chromogenic FIX assays only recently became available but consistently provide different results for some of the rHFIX products versus the one-stage clotting FIX assays; discrepancies also have been reported between FVIII chromogenic and one-stage clotting FVIII assays for recombinant factor replacement products and for gene therapy products.[13,16-20] At present, one-stage clotting assays are used in clinical practice to measure the labeled potency of approved FIX therapeutics and to monitor individuals undergoing therapy.[14,17] With the advent of gene therapy via recombinant adeno-associated viral vectors for hemophilia, assessment of factor activity following vector administration is a key outcome of interest for the assessment of therapeutic efficacy; therefore, the understanding and quantification of assay discrepancies are important.

In the current analysis, plasma samples from subjects receiving fidanacogene elaparvovec showed a strong correlation between measurements from local laboratories and the central laboratory. However, consistently lower values were obtained with the FIX:C assay used at the central laboratory (Actin FSL reagent on BCS XP instrument). When first noted, an investigation of sample handling, shipping, and stability was performed, and variations in sample handling, shipping, and stability were ruled out as causes of the noted differences. The lower activity levels were confirmed in the comparison of commonly used IVD-approved FIX:C one-stage clotting assays in North America. The APTT reagent-dependent differences observed in our results showed the lowest recovery for FIX-Padua transgene product and rHFIX-Padua protein for the Actin FSL one-stage clotting assay, which uses lipids derived from both soy and

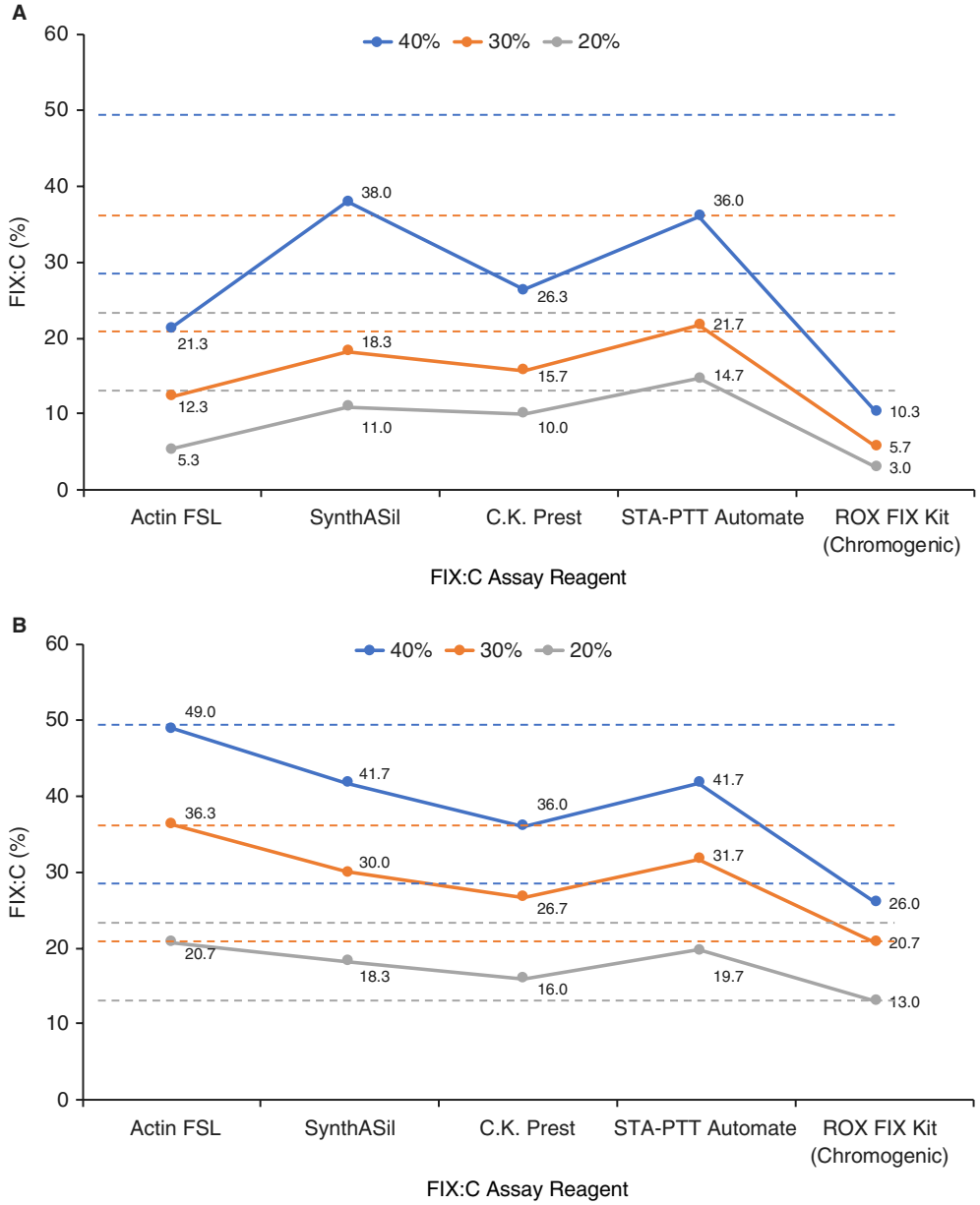

FIGURE 3 FIX:C measured in FIX-deficient plasma spiked with (A) rHFIX-Padua protein and with (B) rHFIX-nonacog alfa. Dashed lines represent the ±25% limits for results for each approximate sample concentration (40%, 30%, and 20%). FIX, factor IX; FIX:C, coagulant activity of factor IX; rH, recombinant human

rabbit brains. The use of the chromogenic assay resulted in the lowest determined FIX:C of the two FIX proteins. The reasons for these differences are not clear at this point. Samelson-Jones et al. have shown that the enhanced activity of recombinant and transgene-expressed FIX-Padua is critically dependent on the interaction between FIXa and FVIIIa; thus, differing efficiencies of generating FIXa or FVIIIa by different activator reagents may lead to larger discrepancies when measuring FIX-Padua.[4] The hyperactivity of FIX-Padua likely facilitates the detection of small differences between one-stage clotting assays.

The impact of specific differences in assay methodologies, instruments, and reagents on the measurement of FIX-Padua activity is not known at this time and could not be determined from the analyses of limited subject samples. Clinical correlation of subject outcomes with determined FIX:C will be important. Preliminarily, clinical outcomes appear to correlate with the one-stage clotting assay results.[2,21] However, these differences suggest that FIX:C assay selection may also be an important consideration when measuring FIX-Padua activity. The differences in the determined FIX-Padua FIX:C among the reagents are likely attributable to the inherent biochemistry of the Padua protein. The similarities in assay-specific FIX:C one-stage clotting and FIX chromogenic results between the transgene, recombinant Padua products and the analysis of samples of the original family possessing the FIX-Padua mutation indicate that assay-based differences are not specific to gene therapy–derived or IVD FIX-Padua.[22] Given the high percentage of

FIX gene therapies currently in development using the Padua mutation in their transgene,[1] a better understanding of FIX:C assay discrepancies and assay performance for measuring response to these FIX gene therapies will be critical factors for successful monitoring gene therapy in individuals with hemophilia B.

DATA SHARING STATEMENT

Upon request, and subject to certain criteria, conditions and exceptions (see https://www.pfizer.com/science/clinical-trials/trial-data-and-results for more information), Pfizer will provide access to individual deidentified participant data from Pfizer-sponsored global interventional clinical studies conducted for medicines, vaccines, and medical devices (1) for indications that have been approved in the United States and/or European Union or (2) in programs that have been terminated (ie, development for all indications has been discontinued). Pfizer will also consider requests for the protocol, data dictionary, and statistical analysis plan. Data may be requested from Pfizer trials 24 months after study completion. The deidentified participant data will be made available to researchers whose proposals meet the research criteria and other conditions, and for which an exception does not apply, via a secure portal. To gain access, data requestors must enter into a data access agreement with Pfizer.

ACKNOWLEDGMENTS

Medical writing and editorial support were provided by Michael Morren, RPh, MBA, of Peloton Advantage, LLC, an OPEN Health company, and funded by Pfizer Inc.

CONFLICT OF INTEREST

Dr. Robinson and Dr. Tiefenbacher are employees of Laboratory Corporation of America, which was hired by Spark Therapeutics and by Pfizer Inc. to perform testing for this study. Dr. George serves as a principal investigator for gene therapy trials sponsored by Spark Therapeutics and Pfizer Inc. Dr. Carr is an employee of Spark Therapeutics and may own stock/options in the company. Dr. Samelson-Jones serves as a clinical investigator for gene therapy trials sponsored by Spark Therapeutics and Pfizer Inc. Dr. Arruda receives research support and consulting fees from Pfizer Inc. and is the recipient of the Aspire Grant from Pfizer Inc. for unrelated research on FIX variants. Drs. Murphy, Rybin, and Rupon are employees of Pfizer Inc. and may own stock/options in the company. Dr. High was an employee of, and previously held equity in, Spark Therapeutics.

AUTHOR CONTRIBUTIONS

Stefan Tiefenbacher, Lindsey A. George, Benjamin J. Samelson-Jones, Marcus E. Carr, Katherine A. High, and Valder R. Arruda contributed to the study design of the phase 1/2a study. Stefan Tiefenbacher and Mary M. Robinson designed the one-stage clotting assay and chromogenic substrate assay experiments outside of this clinical study, in collaboration with Marcus E. Carr and Katherine A. High. Benjamin J. Samelson-Jones and Lindsey A. George were study investigators and Benjamin J. Samelson-Jones enrolled subjects. Mary M. Robinson, Stefan Tiefenbacher, and Katherine A. High collected and assembled data for the one-stage clotting assay and chromogenic substrate assay experiments outside of the clinical study. Data analysis: John E. Murphy, Denis Rybin, Jeremy Rupon, Mary M. Robinson, Stefan Tiefenbacher, Benjamin J. Samelson-Jones, Katherine A. High, and Lindsey A. George participated in data analysis. All authors contributed to manuscript preparation, with the support of medical writers provided by Pfizer Inc. All authors had full access to the data and participated in data interpretation and the critical review and revision of the manuscript. All authors approved the manuscript for submission.

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How to cite this article: Robinson MM, George LA, Carr ME, et al. Factor IX assay discrepancies in the setting of liver gene therapy using a hyperfunctional variant factor IX-Padua. *J Thromb Haemost*. 2021;19:1212–1218. https://doi.org/10.1111/jth.15281