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ORIGINAL ARTICLE

Performance of factor IX extended half-life product measurements in external quality control assessment programs

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Abstract

Background: Patients with hemophilia B are increasingly treated with extended half-life (EHL) factor IX (FIX) concentrates. For the laboratory, introduction of these EHL concentrates presents a major challenge. To understand the variation in FIX activity levels, all available diagnostic assays need to be directly compared.

Methods: The ECAT, UKNEQAS, and RCPAQAP have collaboratively performed a global survey to evaluate the quality of FIX measurements using FIX deficient plasma samples spiked with recombinant FIX (rFIX), rFIXFP, rFIXFc, and N9-GP to levels at typical FIX trough (6 IU/dL) and peak levels (60 IU/dL). Participants were asked to use their routine protocols, using one-stage assays (OSA) or chromogenic assays (CA).

Results: In samples spiked with 6 IU/dL product, median (25%-75% range) FIX activity levels (OSA), were 8.0 IU/dL (7.0-9.2) for rFIX, 6.0 IU/dL (4.0-7.1) for rFIXFP, 6.6 IU/dL (5.5-8.0) for rFIXFc, and 4.9 IU/dL (3.5-8.4) for N9-GP. In samples spiked with 60 IU/dL, FIX activity levels measured (using OSA) was 63.0 IU/dL (59.9-67.0) for rFIX, 42.5 IU/dL (28.2-47.0) for rFIXFP, 50.0 IU/dL (45.0-55.0) for rFIXFc, and 34.0 IU/dL (24.8-67.5) for N9-GP. Considerable differences were observed between reagents for all samples. With CA, there was also quite some variation, but no differences between reagents.

Conclusion: Large variation is observed in the measurement of FIX activity levels after administration of rFIX and EHL FIX products. For N9-GP, most silica-based assays show especially high levels. It is essential to standardize and improve reliability of measurements of these concentrates as diagnosis and treatment monitoring is based on these results.

KEYWORDS

extended half-life products, external quality control, factor IX, hemophilia B, laboratory diagnosis

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1 | INTRODUCTION

Patients with hemophilia B, an X-linked recessive bleeding disorder caused by deficiency of coagulation factor IX (FIX), have a high risk of (spontaneous) bleeding. These patients are treated prophylactically or on demand in case of bleeding with replacement therapy eg, FIX concentrates. Traditionally, the FIX concentrates were purified from human plasma. Since 1997 recombinant FIX (rFIX) is used to treat patients, minimizing risks of infections with blood-transferred diseases.[1] The latest recent therapeutic innovations include development of extended half-life (EHL) concentrates.[2-4] Different technologies have been used to prolong FIX half-life: for rFIXFP (Idelvion®) FIX albumin fusion was performed, for rFIXFc (Alprolix®) FIX-Fc fragment IgG1 fusion, and for N9-GP (Refixia®/Rebinyn®) on glycopolyethylene glycol (PEG)ylation (active component: nonacog beta pegol) was applied.5 The introduction of EHL concentrates may improve quality of life by reducing injection frequency from several times weekly with standard half-life (SHL) concentrates to once weekly.[5,6]

For the laboratory, introduction of FIX EHL concentrates presents a major challenge, as these concentrates often behave differently in the assays that determine FIX levels than the traditional SHL products. At the moment, the available FIX assays are based on two principles. The first, and most-used type of diagnostic assay for FIX levels, is the one-stage clotting activity (OSA) measurement, an activated partial thromboplastin time (aPTT)-based assay. These assays mainly use either ellagic acid, silica, or kaolin as activators, and these activators give similar results when we use them to measure FIX levels in a diagnostic setting in most cases However, different results have also been reported for some of these OSA assays.[7-16] Also, in external quality assessment programs, such as external quality control of diagnostic assays and tests (ECAT), UKNEQAS (blood coagulation), and the Royal College of Pathologists of Australasia Quality Assurance Program (RCPAQAP) in which plasma FIX is analyzed in diagnostic surveys, no large differences have been demonstrated between activators, aPTT reagents, deficient plasmas, and applied instruments. The alternative method is the chromogenic two-stage method (CA), with a first stage in which the generation of activated factor X (FXa) depends on the activity of the plasma FIX, and a second stage in which color is generated by FXa from a chromogenic substrate. The guideline on the declaration of the quantitative composition/potency labeling of biological medicinal products that contain modified proteins as active substance published by the European Medicine Agency (EMA) and effective from September 2014 indicates that potency assays for each modified coagulation factor concentrate should be based on in vitro and in vivo clinical studies of assay methods involving multiple methods and reagents and that labeling can be in international units (IU) based on statistical validity when assayed against the relevant International standard.[17] This is also recommended by Scientific and Standardisation Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) on potency labelling of factor VIII and factor IX concentrates.[18] Several studies have shown that the different EHL FIX concentrates show different results with the different assays, which may result in both under- or overestimation of FIX. This is seen for OSA assays with different activators but also between OSA and CA.[16,19-26] A workshop involving the European Medicine Association (EMA) and manufacturers underlined concerns with regard to assay discrepancies in modified SHL and EHL concentrates and provided both information and advice to manufacturers and clinical laboratories regarding how to approach potency labeling and to perform reliable clinical monitoring.[27]

Essentials

- The introduction of extended half-life concentrates for factor IX (FIX) presents a major challenge for the diagnostic laboratory.
- External Quality Organisations (ECAT, UKNEQAS, and RCPAQAP) have performed a global survey to evaluate the quality of FIX measurements using FIX deficient plasma samples spiked with recombinant FIX (rFIX) and extended half-life (EHL) concentrates for FIX.
- Large variation is observed in the measurement of FIX activity levels in samples spiked with rFIX and EHL FIX products, both with one-stage and chromogenic assays.
- There is a need to standardize and improve the measurement of FIX concentrates, because diagnosis and treatment monitoring is based on these results.

However, a comprehensive field study in which a wide variety of available diagnostic assays are directly compared has not yet been performed. Such a study is essential in understanding the variation in measured FIX activity level in patients using EHL concentrates and to manage differences between laboratories as this will affect the introduction and safety of clinical protocols. Furthermore, it is essential to establish analytical performance of the various FIX assays for FIX EHL concentrates currently available.

To obtain a comprehensive overview of the analytical variation of the measurement of the plasma FIX activity using a wide range of commercially available FIX OSA and CA assays, we performed a global study in which the ECAT Foundation, UKNEQAS BC, and RCPAQAP collaborated in determining the analytical quality of FIX activity measurements in plasma samples spiked with rFIXFP, rFIXFc, and N9-GP to levels that represent FIX trough (6 IU/dL) and peak (60 IU/dL) levels. Participants in the external quality control programs were asked to use their routine protocols. This study will gain insight into the underlying laboratory methods that may explain discrepancies.

2 | MATERIALS AND METHODS

2.1 | Study design

Invitations to participate in this study were sent to all FIX participants of the external quality control programs from the non-profit organizations ECAT (n = 175), UKNEQAS (n = 91), and RCAPQAP (n = 18). Results from spiked FIX samples were received from hemostasis

laboratories in 27 countries worldwide (Argentina, Australia, Belgium, Canada, Croatia, Denmark, Finland, France, Greece, Hong Kong, Hungary, Ireland, Israel, Italy, Malta, Netherlands, New Zealand, Poland, Portugal, Spain, Serbia, South Africa, Sweden, Switzerland, Thailand, United Kingdom, and United States) from ECAT (n = 64),[28,29] UKNEQAS (n = 82), and RCAPQAP (n = 18). Participating laboratories were asked to perform FIX activity assays on each of the spiked samples, using their usual reagents and instruments for measuring FIX activity when monitoring post FIX concentrate infusion. Laboratories were asked, if possible, to perform both OSA and CA on these samples. Participants filled in a questionnaire for detailed information on, eg, assay methodology, instruments, reagents, and calibrator used. Participants were included if they reported at least one result of a measurement (n = 172).

2.2 | Study samples

Factor IX (FIX) deficient plasma from a severe hemophilia B patient without detectable FIX (George King Biomedical Inc) was spiked with one of the FIX concentrates (rFIXFP, rFIXFc, N9-GP, and rFIX [Benefix]). The absence of FIX in the deficient plasma was confirmed prior to spiking (<1 IU/dL). Each concentrate was spiked in two different concentrations (6 and 60 IU/dL). The concentration of the FIX products used was the potency label as defined by the pharmaceutical company. Concentrates often carry a nominal potency such as 500 or 1000 IU as well as a more accurate assayed potency. The assayed potency (rather than nominal) was used for calculations of dilution factor to construct the 60 IU/dL samples. The 6 IU/dL samples were constructed by a 1 in 10 dilution of the 60 IU/dL sample using the same FIX deficient plasma as diluent. Plasma samples were then aliquoted and lyophilized as previously described by UKNEQAS before distribution.[30]

2.3 | Laboratory methods

The participating laboratories used either an OSA or a CA and in some cases used both. A wide variety of reagents and instruments were used (commercial and homemade). For the OSA, the majority of participants used IL HemosIL Synthasil, Siemens Actin FS, Siemens Pathromtin SL, Stago Cephalin/Kaolin/CK Prest as aPTT reagents and Werfen/IL ACL, Siemens BCS/BCS-XP/BCT, Stago STA, and Sysmex as instruments. For the CA, the Rossix kit and the Hyphen kit were similarly used among laboratories.

2.4 | Statistical analysis

Data are presented as median, range, and coefficient of variation of the values and the median of the recovery, based on the potency assigned to the sample. Some participants filled in information on their methods, but only those participants were included in the analyses who also submitted measured values. All reported data are used in the analyses. Statistical analysis was performed using IBM SPSS Statistics version 24.

TABLE 1 Reagents used by the participants in the survey

Assay type	N
One-stage assays	160
Silica	102
IL HemosIL Synthasil	68
Siemens Pathromtin SL	12
Stago PTT (automate)	9
IL HemosIL APTT-SP	4
Tcoag TriniCLOT APTT S	4
Tcoag TriniCLOT APTT HS	4
Stago PTT-LA	1
Polyphenolic	44
Siemens Actin FS	32
Siemens Actin FSL	5
Stago Cephascreen	4
Sekusiu Coagpia APTT-N	1
IL HemosIL SynthAFax	1
Diagnostic Grifols APTT synth	1
Kaolin	14
Stago Cephalin/Kaolin/CK Prest	14
Chromogenic two-stage assays	41
Hyphen	21
Rossix	19
Home made	1

3 | RESULTS

3.1 | Reagents and instruments

The OSA was performed by 93% of the laboratories (160/172) who submitted at least one result; 120 laboratories only performed the OSA, while 35 laboratories reported both the OSA and the CA. In total 41 laboratories performed the CA of which 35 reported results for both assays and six laboratories only the results of the CA (Table 1).

For the CA, the two available commercial kits (Hyphen kit and the Rossix kit), were used by 21 and 19 participating laboratories, respectively. Furthermore, 1 participant used an in-house chromogenic assay (Table 1).

3.2 | One-stage clotting assay

In samples spiked with 6 IU/dL product, the median (25%-75% range) FIX activity values were 8.0 IU/dL (7.0-9.2; median recovery 133%) for rFIX, 6.0 IU/dL (4.0-7.1, 100%) for rFIXFP, 6.6 IU/dL (5.5-8.0,

110%) for rFIXFc, and 4.9 (3.5-8.4, 82%) IU/dL for N9-GP (see Figure 1A). At 6 IU/dL, the between-laboratory variation (CV, coefficient of variation) was in the same range for rFIX (CV 25%), rFIXFP (CV 41%), and rFIXFc (CV 36%), and with N9-GP we saw very large between-laboratory variation of 169%.

In samples spiked with 60 IU/dL, the median (25%-75% range) FIX activity was 63.0 IU/dL (59.9-67.0, median recovery 105%) for rFIX, while all FIX-EHL concentrates were underestimated with the OSA with 42.5 IU/dL (28.2-47.0, 71%) for rFIXFP, 50.0 IU/dL (45.0-55.0, 85%) for rFIXFc, and 34.0 IU/dL (24.8-67.5, 57%) for N9-GP (Figure 1B). For the samples spiked with 60 IU/dL, the between-laboratory variation (CV) was 12% for rFIX, 30% for rFIXFP, and 19% for rFIXFc. Also with the high dose, N9-GP showed a large between-laboratory variation (CV 179%; Figure 1B).

A wide variation of reagents and instruments were used for the OSA. The aPTT activating reagents used among laboratories were

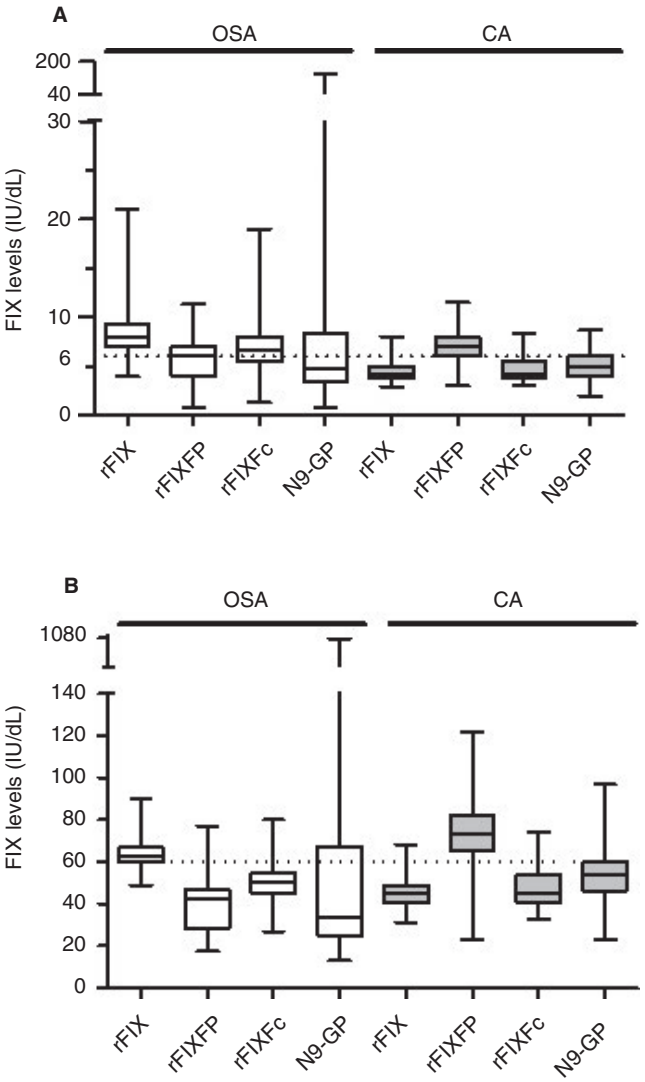

FIGURE 1 Boxplots of reported factor IX (FIX) levels (mean and standard deviation) for below typical trough levels of 6 IU/dL (Panel A) and peak levels of 60 IU/dL (Panel B) with one-stage and chromogenic FIX assays

silica (102 laboratories, 64%), ellagic acid (40 laboratories, 25%), kaolin (14 laboratories, 9%), and polyphenols (4 laboratories, 3%; Table 1). aPTT reagents that were used by the participating laboratories used different activators, namely silica (n = 102; Tcoag TriniCLOT APTT S, Tcoag TriniCLOT APTT HS, Stago PTT-LA, Stago PTT (automate), Siemens Pathromtin SL, IL HemosIL Synthasil, and IL HemosIL APTT-SP), ellagic acid (n = 40; Siemens Actin FSL, Siemens Actin FS, Sekusiu Coagpia APTT-N, IL HemosIL SynthAFax, and Diagnostic Grifols APTT synth), cephalin/kaolin (n = 14; Stago Cephalin/Kaolin/CK Prest), or cephalin/polyohenolic (n = 4; Stago Cephascreen; Table 1).

Large between-laboratory differences in reported levels were seen for all FIX concentrates and with all different aPTT reagents at 6 IU/dL (Figure 2A-D and Tables 2 and S1 in supporting information) and at 60 IU/dL (Figure 3A-D and Tables 3 and S1).

Considerable differences were observed between reagents, both for the low-dose and the high-dose samples, without there being a clear pattern. The mean FIX activity levels measured for rFIX were rather similar for all aPTT reagents for the low-dose sample (Figure 2A), but for the high-dose sample more variation was observed (Stago PTT Automate, Tcoag TriniClot APTT-S, Siemens Actin FSL, and Stago Cephscreen showed higher median values; Figure 3A). Also for the low-dose rFIXFP similar median values were found for all aPTT reagents, except for IL HemosIL Synthasil and Siemens Pathromtin SL, which demonstrated slightly higher median values (Figure 2B). For the high-dose sample an underestimation of rFIXFP was observed with all aPTT reagents, except with Stago Cephascreen (Figure 3B). For rFIXFc similar results were obtained with the low-dose sample for almost all aPTT reagents. Only Stago APTT Automate gave slightly lower and Siemens Actin FSL slightly higher median values (Figure 2C). For the high dose of rFIXFc an underestimation was measured with most of the aPTT reagents, except for Siemens Acin FS, Siemens Actin FSL, and Stago Cephascreen (Figure 3C). For N9-GP, significant differences were shown between different aPTT reagents, with strong overestimation for all silica-based aPTT reagents, except IL (HemosIL Synthasil; Figures 2D and 3D).

There was very limited variation in the combination of aPTT reagents and instruments, with 93% using both from the same company and only single laboratories using specific divergent combinations. Therefore, it was impossible to study the individual contribution of reagents and instruments to the between-laboratory variation. Also, 80% of the laboratories used the calibrator from the same company as reagents and instrument. Other combinations were usually single laboratories, and therefore it was not possible to study the unique contribution of the calibrator.

3.3 | Chromogenic assay

Reported FIX activities for each product as measured with the CA are presented in Figure 1. In samples spiked with 6 IU/dL product, the median (25%-75% range) FIX activity values were 4.2 IU/dL

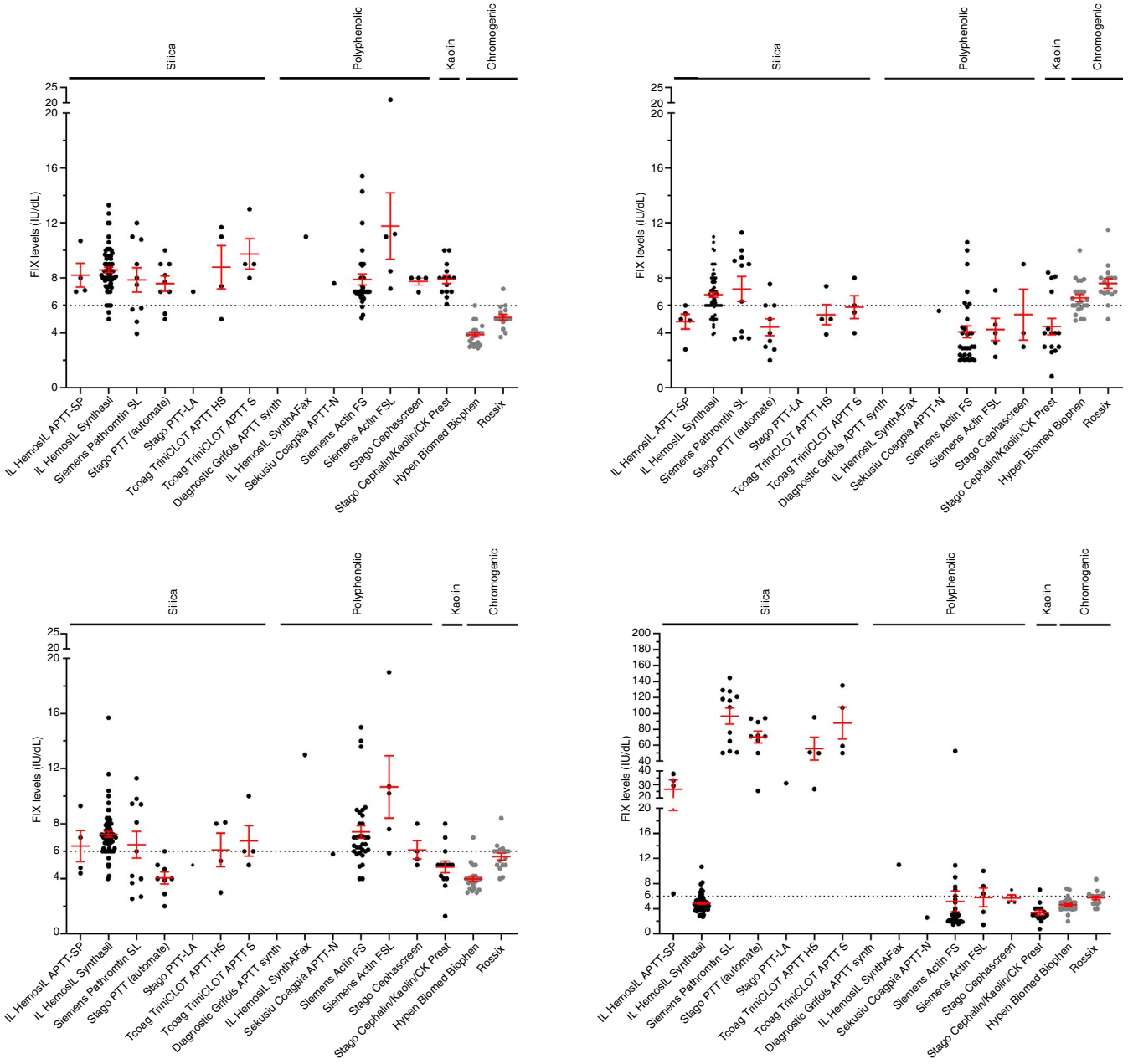

FIGURE 2 Reported factor IX (FIX) levels (all reported values, mean and standard deviation) for below typical trough levels (6 IU/dL) with one-stage assays and chromogenic assay FIX reagents. A, rFIX; B, rFIXFP; C, rFIXFc; D, N9-GP. The Y-axis is broken to improve the presentation when there is a wide variation in results

(3.8-5.0; median recovery 70%), 7.0 IU/dL (6.0-7.9, 117%), 4.2 IU/dL (3.8-5.5, 70%), and 5.0 IU/dL(4.0-6.0, 83%) for rFIX, rFIXFP, rFIXFc, and N9-GP, respectively (Figure 1A).

In samples spiked with 60 IU/dL, the median (25%-75%) absolute FIX activity values were 45 IU/dL (40.5-79.0; median recovery 75%); 73.3 IU/dL (65.3-81.9, 122%); 45.0 IU/dL (40.8-54.0, 75%); and 53.6 IU/dL (46.2-60.0, 89%) for rFIX, rFIXFP, rFIXFc, and N9-GP respectively (Figure 1B).

Both for the 6 and 60 IU/dL sample the between-laboratory variation (%CV) was similar for rFIX, rFIXFP, rFIXFc, and N9-GP (6 IU/dL: 25.4%, 21.8%, 26.5%, and 25.8%, respectively; 60 IU/dL: 17.6%, 22.2%, 19.7%, and 22.8%).

Significant differences were observed between the Hyphen and Rossix method for rFIX at both levels, rFIXFc at high levels, and N9-NP at low levels; the other results were not significantly different (Figures 2A-D and 3A-D).

One determinant of the measured levels may be the calibrator used. Different calibrators were used for both chromogenic assays (Table S2 in supporting information), and for the high-dose sample significant differences were observed in the reported values. For rFIXFP, the reported values were higher than expected, and higher than for the other products, where reported values were lower than expected (Table S3 in supporting information). Also, within a product, we saw differences. With the same product we

TABLE 2 One-stage clotting and chromogenic assays: results by aPTT activator, levels set at 6 IU/dL

	rFIX	rFIXFP	rFIXFc	N9-GP
OSA				
Silica				
Median (IU/dL)	8.3	6.2	7.0	5.4
CV (%)	21.7	29.0	30.7	140
Range (IU/dL)	4.0-13.3	2.0-11.3	2.0-15.7	2.7-144.5
Median recovery (%)	138	103	117	90
Polyphenolic activator				
Median (IU/dL)	7.3	4.0	7.0	3.0
CV (%)	40.0	40.8	51.7	132
Range (IU/dL)	5.1-21.0	2.0-10.6	4.0-19.0	1.47-52.7
Median recovery (%)	122	66	117	50
Cephalin/kaolin				
Median (IU/dL)	8.0	3.5	5.0	3.0
CV (%)	14.9	50.7	32.5	43.4
Range (IU/dL)	6.1-10.0	0.8-8.1	1.3-8.0	0.8-7.0
Median recovery (%)	133	58	83	50
Chromogenic assays				
Hyphen				
Median (IU/dL)	3.8	6.3	3.8	4.3
CV (%)	14.6	14.2	13.5	19.4
Range (IU/dL)	2.9-5.0	4.9-8.0	3.0-5.0	2.0-6.0
Median recovery (%)	63	105	63	72
Rossix				
Median (IU/dL)	5.0	7.5	5.5	6.0
CV (%)	16.8	20.3	19.5	22.7
Range (IU/dL)	3.7-7.2	5.0-11.5	4.0-8.4	3.0-8.7
Median recovery (%)	83	125	92	100
In house				
Level (IU/dL)	8.0	3.0	4.0	3.0
Recovery (%)	133	50	67	50

found different levels depending on the calibrator that was used (Table S3).

4 | DISCUSSION

In this global study on the analytical quality of measuring FIX activity in plasma samples spiked with rFIXFP, rFIXFc, and N9-GP to levels that represent FIX trough (6 IU/dL) and peak (60 IU/dL) levels, our main observations were that the reported values show a large variation both between products but also between laboratories. In addition, significant differences were demonstrated between reagents used in the OSA, especially in measuring N9-GP.

An important characteristic of this study is that our results reflect the real-life situation in laboratories that measure FIX activity. The study includes many laboratories from all over the world, using the available range of diagnostic assays and instruments. Centers used the assay design that would be applied when monitoring patients. At the same time, we realize that we live in a constantly changing world and new and improved reagents and instruments are becoming available all the time. This emphasizes the need for continuous post-marketing surveillance as performed through proficiency testing programs.

Measurement of FIX levels in patients treated with EHL concentrates is frequently needed as tailoring of dosing of the patients is increasingly based on the results, as applied in pharmacokinetic-guided dosing for example. Undertreatment of patients may result in bleeding complications and should therefore be avoided. Differences between assays, leading to different FIX activity recovery levels, will have impact on dosing regimens. However, we should be aware data is scarce on associations between FIX activity levels and bleeding risk.

In our study, levels measured in the samples spiked with rFIX were also not showing optimal recoveries. The recovery was increased in the 6 IU/dL sample with OSA and decreased with CA,

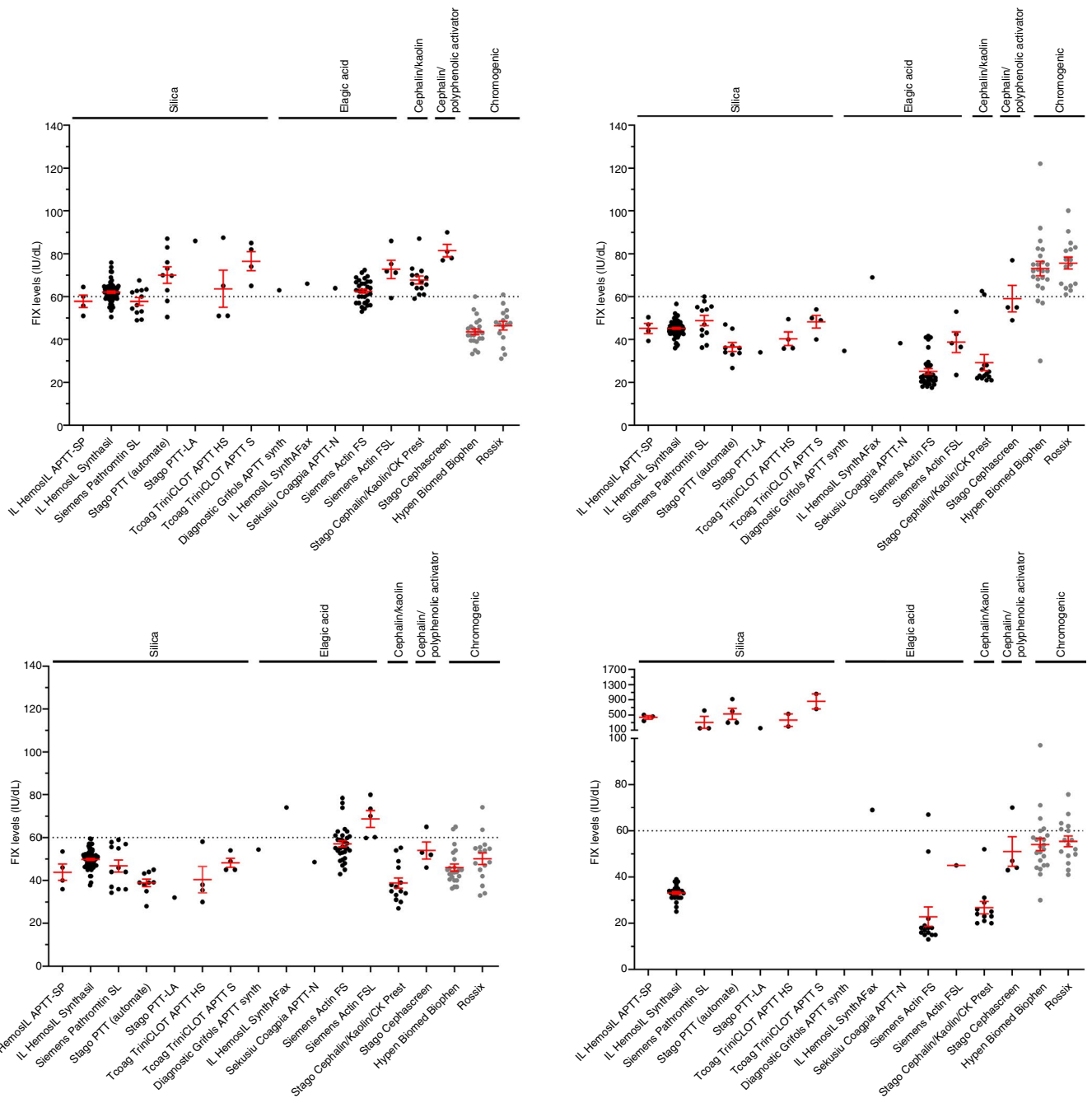

FIGURE 3 Reported factor IX (FIX) levels (all reported values, mean and standard deviation) for high levels (60 IU/dL) with one-stage assays and chromogenic assay FIX reagents. A, rFIX; B, rFIXFP; C, rFIXFc; D, N9-GP. The Y-axis is broken to improve the presentation when there is a wide variation in results

while it was slightly increased in the 60 IU/dL sample with OSA and also decreased with the CA. Previously, Sommer et al have also shown increased recovery with rFIX by OSA.[8] In this study, the mean CV of the OSA were 44% and 31% for the low and high values, respectively, and 30% and 19% when we did not consider the large variation in N9-GP samples. In proficiency testing surveys utilizing samples containing native FIX these percentages are usually lower (around 20%-25% and 10% for low and normal values in ECAT and UKNEQAS regular EQA surveys). This indicates that, except for certain specific reagent effects with some products, the between-laboratory variation of EHL products is similar to that for the measurement of FIX in patients.

Also in this study, the lowest CVs occurred for the standard half-life product. This suggests that all tested modifications on FIX that prolong their half-life in the products studied have some effect on OSA. The most dramatic effect was seen for N9-GP, where either lower levels were measured or extremely high levels. This difference could not be explained solely by the use of the class of activator, although all but one reagent using silica as activator showed very high FIX levels. It should be noted that there could be different forms

TABLE 3 One-stage clotting assay: results by aPTT activator, levels set at 60 IU/dL

	rFIX	rFIXFP	rFIXFc	N9-GP
OSA				
Silica				
Median (IU/dL)	62.0	45.0	48.5	35.0
CV (%)	12.4	12.8	14.3	141.5
Range (IU/dL)	49.0-87.5	26.7-60.0	28.0-59.5	25.0-1060
Median recovery (%)	103	75	81	58
Polyphenolic				
Median (IU/dL)	64.3	24.0	56.5	18.5
CV (%)	12.8	23.6	15.2	34.0
Range (IU/dL)	53.0-90.0	17.5-77.0	43.0-80.0	13.0-70.0
Median recovery (%)	107	40	94	31
Kaolin				
Median (IU/dL)	66.5	23.5	36.0	24.0
CV (%)	10.3	47.9	22.9	34.0
Range (IU/dL)	59.0-87.0	21.0-62.6	27.0-55.3	20.0-52.0
Median recovery (%)	111	39	60	40
Chromogenic assays				
Hyphen				
Median (IU/dL)	42.0	72.0	43.8	52.0
CV (%)	10.2	22.9	14.4	24.5
Range (IU/dL)	33.3-52.0	30.0-122.0	36.3-64.0	30.0-97.0
Median recovery (%)	70	120	73	87
Rossix				
Median (IU/dL)	47.7	77.2	53.0	56.0
CV (%)	18.8	15.0	21.1	17.2
Range (IU/dL)	31.0-60.9	58-100.10	33.0-74.1	41.0-75.7
Median recovery (%)	80	129	88	93
In house				
Level (IU/dL)	68.0	23.0	35.0	23.0
Recovery (%)	113	38	58	38

of silica in different reagents that affect such relationships. Rosen et al studied the behavior of N9-GP in the aPTT assay. They observed that N9-GP, in the presence of one form of silica, is prematurely converted to activated FIX (FIXa) during the contact activation phase of the clotting assay by activated FXI (FXIa) and plasma kallikrein (PK). They conclude that this phenomenon leads to the overestimation of the FIX activity in the OS clotting assay. This effect was not seen for native FIX.[20] Also, Tiefenbacher et al did not see this.[21]

For the chromogenic assays the recovery percentages were slightly lower with Hyphen reagents than with Rossix. Both reagents measured median recovery higher than 100% for rFIXFP and below 100% recovery for the other drugs.[27] The assignment of potency to FIX products in Europe and North America is done by the one-stage assay. This is prescribed for both standard and EHL products although the full reagent details, such as aPTT reagent, are not available in the literature for all products to the best of the authors' knowledge. Also, Tiefenbacher et al did not see this.[21] One explanation may be that we prepared our samples differently. We used the assayed potency value on the individual vials that were used to prepare the spiked samples in our calculations.

Another determinant may be the calibrator that was used. In our study it was not possible to evaluate this, because almost all laboratories used calibrator, reagent, and instrument from the same company. For the chromogenic assay this relationship was less tight and we could evaluate the different results for the four most-used calibrators. With the calibrators that were used within the chromogenic

assays, we see that the mean values for the high samples are lower than expected, based on the assigned value, for rFIX, rFIXIc, and N9-GP, while with all calibrators rFIXFP was higher than expected (Table S3). Also, within products, significant differences were observed, with the lowest levels for all of the products measured using the Precision Biologic Normal Reference plasma.

It should also be considered that in this study we used spiked samples. This has some limitations compared to samples from patients using the medication. It is our experience (unpublished results) that samples with the products we now studied appear to be commutable. Furthermore, the potency assignment to product vials is based on OSA and is based on OSA using a specific aPTT reagent/analyzer protocol which is unlikely to match all protocols used by the study participants. Further, differences in methods of potency assignment between drugs may add to the observed variability found in this study. However, this assigned potency is what the doctors work with when deciding on the treatment of the patients.

In conclusion, a large variation is observed in FIX activity measurement of EHL FIX concentrates using both OSA and CA assays, with over- and underestimation of the measured levels, without a clear pattern. Special attention should be given to patients on N9-GP, as most silica-based assays give very high levels. It is essential to standardize FIX activity level measurement of EHL concentrates and to employ and develop assays that accurately measure the plasma FIX activity in patients using these products.

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CONFLICTS OF INTEREST

Angelique Nederlof has nothing to disclose. Steve Kitchen has received speaker/consultancy fees from Bayer, NovoNordisk, Pfizer, Sobi, Takeda. Piet Meijer received fees for meetings of advisory board meetings of Novo Nordisk and Grifols Diagnostica and speakers fee from Shire. Marjon H. Cnossen has received unrestricted research grants for investigator-initiated studies and educational as well as travel grants from Pfizer, Baxalta/Shire/Takeda, Bayer, CSL Behring, Novo Nordisk, Novartis, Nordic Pharma, and for advisory board activities from Bayer, Roch,e and Novartis. Nae Ali Pour has nothing to disclose. Geoffrey Kershaw has nothing to disclose. Ian Jennings has received speaker fees from Pfizer. Isobel Walker has nothing to disclose. Moniek PM de Maat received speaker fees and unconditional research contributions from Werfen, Siemens, Roche, and Stago.

AUTHOR CONTRIBUTIONS

A. Nederlof analyzed the data and contributed to the writing of the manuscript. S. Kitchen designed the study and contributed to the writing of the manuscript. P. Meijer designed the study and contributed to the writing of the manuscript. M. Cnossen supervised the PhD student and reviewed the manuscript. N. Ali Pour designed the study and contributed to the writing of the manuscript. G. Kershaw contributed to analyzing the data and reviewed the manuscript. I. Jennings prepared and distributed samples, and organized collection and analysis of the data. I. Walker designed the study and contributed to the writing of the manuscript. M. P. M. de Maat contributed to the analysis of the data and the writing of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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