Received: 26 September 2019 | Revised: 20 November 2019 | Accepted: 3 December 2019

DOI: 10.1111/hae.13915

Haemophilia WILEY

ORIGINAL ARTICLE

FIX potency of rFIX-Albumin fusion protein is underestimated by one-stage methods using silica-based APTT reagents

Steffen Rosén[1] | Pia Bryngelhed[2]

[1]Private Consultant, Molndal, Sweden

[2]Rossix AB, Molndal, Sweden

Correspondence
Steffen Rosén, Private Consultant, Hallavagen 12, 42837 Kallered, Molndal, Sweden.
Email: steffen.rosen@rossix.com

Abstract

Introduction: Higher potency is obtained with chromogenic substrate (CS) methods and one-stage (OS) method with SynthAFax vs silica-based OS methods on analysis of albutrepenonacog alpha (rFIX fused with albumin, rFIX-FP).

Aim: Investigation of the effect of contact activator in search for explanation of discrepancy between methods.

Methods: Chromogenic Rox Factor IX method and OS methods with Pathromtin SL, SynthAFax or new OS method variants using different phospholipid emulsions and addition of either colloidal silica to create APTT reagents or addition of human FXIa together with calcium ions, in the latter case omitting contact activation. The effect of (a) adding different amounts of colloidal silica or (b) mixtures of Pathromtin SL and purified phospholipids immediately before addition of FXIa and calcium chloride was also explored. FIX activation via tissue factor/FVIIa was also made.

Results: FIX potency of rFIX-FP when using APTT reagents with pure phospholipid emulsions with added colloidal silica was similar to OS method with Pathromtin SL. In contrast, close to 80% higher FIX potency for rFIX-FP, and similar to OS method with SynthAFax and to the CS method, was obtained when FXIa replaced contact activation. No discrepancies were obtained for plasma-derived FIX. Gradual decrease of colloidal silica or decreasing proportion of Pathromtin SL added just before addition of FXIa raised rFIX-FP potency to that obtained with SynthAFax and Rox Factor IX. Supportive results were obtained with the tissue factor/FVIIa method.

Conclusion: Colloidal silica and Pathromtin SL impair activation of rFIX-FP, causing underestimation of rFIX-FP potency.

KEYWORDS

assay discrepancies, chromogenic assay, factor IX, haemophilia B, one-stage assay, silicon dioxide

1 | INTRODUCTION

Haemophilia B is a congenital bleeding disorder caused by absent or malfunctional coagulation factor IX (FIX). Infusion of FIX concentrate is made prophylactically or on-demand to prevent and stop serious bleeding. In recent years, modified FIX products with an extended half-life (EHL-rFIX) have been approved for therapeutic use. Such products are significantly improving the quality of life for haemophilia B patients by allowing a greater time interval between infusions.

However, FIX potency determination of EHL-rFIX products may show discrepant results between one-stage (OS) and chromogenic substrate (CS) methods[1-3] and non-parallelism vs a plasma calibrator

 © 2020 John Wiley & Sons Ltd

has also been demonstrated, adding further complexity to potency assignments.[4] Both higher and lower OS vs CS FIX potency has been reported for nonacog beta pegol, N9-GP.[5,6] Overassignment of N9-GP by some OS methods using silica-based APTT reagents has been shown to be due to silica-mediated activation of FIX already during contact activation [7] and underassignment when using certain ellagic acid- or silica-based APTT reagents is explained by contact activator-impaired activation.[8]

For rFIX-FP, OS methods show lower FIX potencies than CS methods, the only published exception being an OS method with the ellagic acid-based reagent SynthAFax.[9-12]

In the present study, OS methods with Pathromtin SL and SynthAFax (intrinsic coagulation) have been compared with one CS method in FIX potency determination of plasma-derived (pd) FIX and of rFIX-FP. Furthermore, variant OS methods have been applied in which either colloidal silica or factor XIa (FXIa) was added to purified phospholipids, thus including or excluding contact activation, respectively. The effect of adding different amounts of colloidal silica or different mixtures of Pathromtin SL and phospholipid without any contact activator immediately before addition of FXIa was also investigated. Finally, FIX activation with tissue factor and FVIIa (extrinsic activation) was explored. Part of this work has been published earlier.[13]

2 | MATERIALS AND METHODS

2.1 | FIX sources

Pure pdFIX (Enzyme Research Laboratories), Replenine (Blood Products Laboratory), denoted pdFIX Concentrate below, and albutrepenonacog alpha (Idelvion, rFIX-FP), in which rFIX is fused with human albumin (CSL Behring).

The 4th and 5th International pd FIX Concentrate Reference Standards (07/182 and 14/148, respectively), denoted IS FIX below, were from the National Institute of Biological Standards and Control (NIBSC).

2.2 | APTT reagents and Phospholipid emulsions

Silica-based Pathromtin SL (Siemens Healthineers) and ellagic acid-based SynthAFax (Instrumentation Laboratory). Pathromtin SL is used for potency assignment of rFIX-FP.[10]

Immuno-depleted FIX deficient plasma from Siemens was used in all OS methods.

The phospholipid emulsions phospholipid-TGT (28 mol% phosphatidylserine, 30 mol% egg sphingomyelin and 42 mol% phosphatidylcholine) and phospholipid-PF3 (8 mol% phosphatidylserine, 23 mol% egg sphingomyelin, 27 mol% phosphatidylethanolamine, 40 mol% phosphatidylcholine and 2 mol% Lyso-phosphatidylinositol) were from Rossix. Phospholipid-PF3 has a phospholipid composition similar to Platelet Factor 3.[14]

2.3 | Other materials and reagents

Colloidal Silica Bindzil 309/220 was from AkzoNobel (Bohus). The silica content is 30% by weight and has a particle-specific surface area of 220 m^2/g.

The Rox Factor IX kit (Rossix) was used for chromogenic determination of FIX activity. FIXa activity was determined with the Rox FIX-A kit (Rossix).

The following bioreagents were used: human FXIa (Enzyme Research Laboratories), Tissue factor Recombiplastin 2G (Instrumentation Laboratory) and human FVIIa (Hematological Inc).

0.05 mol/L imidazole buffer pH 7.4 (25°C) with 0.1 mol/L NaCl and 1% bovine serum albumin was used as diluent in OS methods.

0.05 mol/L Tris buffer pH 8.5 (25°C) without NaCl and 1% bovine serum albumin was used for dilution of colloidal silica and of phospholipid emulsions.

Factor IXa Diluent from the Rox FIX-A kit was used for sample and IS FIX dilutions in extrinsic activation of FIX.

Most of the OS analyses were performed on KC-4A Micro (Amelung); some were performed on ACL TOP 500 (IL) and STA-R Evolution (Stago Diagnostica). In all cases, both Pathromtin SL and SynthAFax were used. CS analyses were performed on ACL TOP 500, STA-R Evolution or by a manual microplate method, using the T-Max microplate reader (Molecular Devices).

2.4 | Basic OS methods

50 µL of sample or calibrator (IS FIX) dilution was mixed with 50 µL of FIX deficient plasma. After heating to 37°C, 50 µL of APTT reagent (37°C) was added and contact activation proceeded at 37°C for the prescribed 3 minutes (Pathromtin SL) or 5 minutes (SynthAFax, the latter time also used for OS method variants described below). 50 µL of 25 mmol/L calcium chloride (37°C) was subsequently added to trigger coagulation and the clotting time determined.

2.5 | Variant OS methods

Phospholipid-TGT and phospholipid-PF3 were used as follows:

- Incorporation of 1.8 mL colloidal silica per L phospholipid emulsion to create APTT reagents, using 5 minutes contact activation at 37°C. The stock solution was first prediluted 10-fold with 0.05 mol/L Tris buffer pH 8.5 whereafter 18 µL was added per mL phospholipid emulsion. The chosen concentration of colloidal silica gave calibrator clotting times similar to those obtained with Pathromtin SL
- Incorporation of FXIa in the calcium chloride solution. The FXIa activities were in the range 1.5-6 IU/mL to give clotting times similar to those with colloidal silica in the phospholipid

emulsions. Final phospholipid concentrations were 35 and 100 μmol/L for phospholipid-TGT and phospholipid-PF3, respectively. The relatively lower procoagulant activity of phospholipid-PF3 due to its lower content of phosphatidylserine required a higher final phospholipid concentration in the assay. Similarly, as for contact activation, 4-5 minutes incubation at 37°C of sample, deficient plasma and phospholipid emulsion was used.

- To obtain a direct measure of the influence of colloidal silica, suitably prediluted colloidal silica was added *immediately before* triggering coagulation by addition of FXIa/calcium chloride. In this way, any contact activation was minimized. Potency assignments were only made of rFIX-FP. Five different predilutions of colloidal silica were used to make 0.022, 0.044, 0.088, 0.44 and 2.2 mL of colloidal silica/L phospholipid emulsion, covering a 100-fold range and with the highest concentration corresponding to the same final assay concentration of colloidal silica as above. In this study, phospholipid-TGT was used as the phospholipid source and the final assay concentration was 35 μmol/L.

  The assay was performed as follows: 50 μL of sample or calibrator (IS FIX) dilution was mixed with 50 μL of FIX deficient plasma and 50 μL phospholipid, whereafter the mixture was heated at 37°C. Then, 50 μL of colloidal silica was added, directly followed (time span 7-8 s) by addition of 50 μL of FXIa/calcium chloride to trigger coagulation.

- A mixing study with Pathromtin SL and phospholipid-TGT was performed similarly to the above study. Pathromtin SL was mixed with phospholipid-TGT to provide the same relative contributions of the Pathromtin SL silica contact activator as obtained with added colloidal silica. The content of Pathromtin SL in the phospholipid mixtures was 1%, 2%, 4% and 20%, Pathromtin SL was also tested neat (100%). 50 μL of sample or calibrator dilution was mixed with 50 μL of FIX deficient plasma and heated at 37°C. Then, 50 μL of a mixture of Pathromtin SL and phospholipid-TGT or of neat Pathromtin SL was added, *directly followed* (time span 7-8 s) by addition of 50 μL of FXIa/calcium chloride to trigger coagulation.

2.6 | Activation of FIX by tissue factor and FVIIa (extrinsic FIX activation)

30 μL of FIX sample, diluted in FIXa Diluent was mixed with 150 μL of an activation reagent comprising Recombiplastin 2G, phospholipid-TGT, human FVIIa and calcium chloride. Recombiplastin 2G was reconstituted in 8 mL as prescribed. Final assay conditions: Recombiplastin dilution 1:280, phospholipid 20 μmol/L, FVIIa 0.42 nmol/L and calcium chloride 5.6 mmol/L. rFIX-FP was analysed at two dilutions and IS FIX Concentrate Standard was used as calibrator at four dilutions. Activation proceeded for 30min whereafter subsampling was made into a two-fold excess of ice-cold 5 mmol/L EDTA. After a further three-fold dilution of the stop mixture with FIXa Diluent to obtain a 9-fold total dilution of the subsample, generated FIXa was determined with the chromogenic FIXa kit method as prescribed but with direct consecutive additions of ice-cold Reagent 1 and Reagent 2 (room temperature) to minimize any further FIXa generation. Three independent determinations were made. rFIX-FP potency was derived from the FIX IS standard curve.

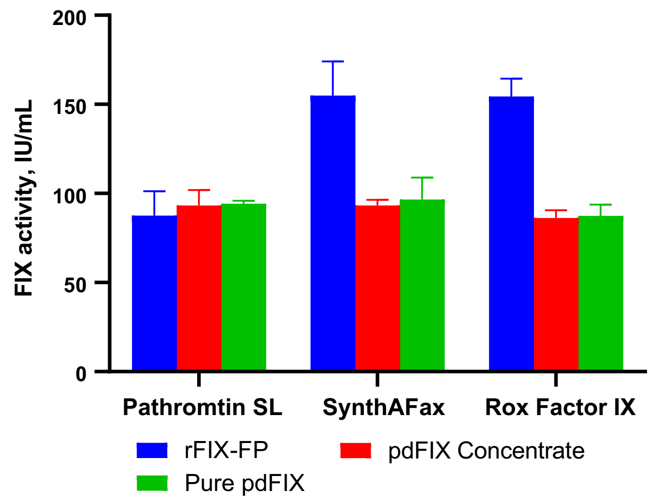

FIGURE 1 Assigned mean potencies of rFIX-FP, pdFIX concentrate and pure pdFIX using OS methods with Pathromtin SL and SynthAFax and the chromogenic Rox Factor IX kit. The 4th or 5th IS FIX Concentrate was used as calibrator. Each result is the mean from two or three dilutions of each sample. The number of determinations was ≥ 3

3 | RESULTS

3.1 | FIX potency determinations

Figure 1 shows that the FIX potencies for both pdFIX sources were similar for the CS method and the two OS methods with Pathromtin SL and SynthAFax. In contrast, FIX potencies for rFIX-FP were distinctly different with potencies divided into two groups. For the CS method and the OS method with SynthAFax, potencies averaged about 155 IU/mL vs about 88 IU/mL with Pathromtin SL.

Figure 2 shows that the two variants of OS methods with each of phospholipid-TGT and phospholipid-PF3 gave similar results for both pdFIX´s, whereas rFIX-FP potencies were again distinctly different. Similar results as with Pathromtin SL were obtained with the two phospholipids containing colloidal silica whereas results in line with SynthAFax and the CS method, about 155 IU/mL, were obtained when FXIa was included with the calcium chloride solution. Notably, in these latter variants, there was no contact activator present.

Similar results were obtained with the two phospholipid emulsions containing either 28 mol% (phospholipid-TGT) or 8 mol% (phospholipid-PF3) phosphatidylserine.

No trend of any change of assigned potencies vs absolute clotting times was noticed (data not shown).

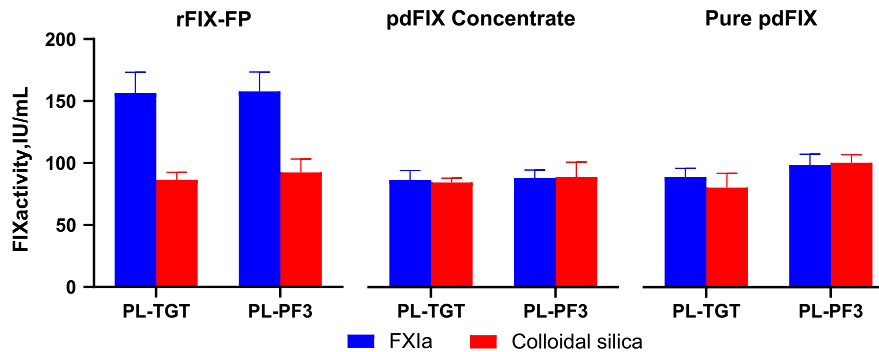

FIGURE 2 Assigned mean potencies of rFIX-FP, pdFIX Concentrate and pure pdFIX using OS methods with phospholipid emulsions phospholipid-TGT and phospholipid-PF3 (for compositions, refer to Materials & Methods) and with (1) 1.8 mL colloidal silica per L emulsion to create APTT reagents and using 5 minutes contact activation or (2) incorporating FXIa with calcium chloride, omitting contact activation. Final phospholipid concentrations were 35 and 100 µmol/L with phospholipid-TGT and phospholipid-PF3, respectively. Final FXIa concentrations varied between 0.25 and 1.5 IU/mL. The 5th IS FIX Concentrate was used as calibrator. Each result is the mean of two or three dilutions of each sample. For each phospholipid source, the same dilution sets of calibrator and samples were used with both colloidal silica and FXIa. The number of determinations of each sample was ≥ 3 with colloidal silica-containing phospholipids. For phospholipids with FXIa, ≥8 determinations were made of rFIX-FP and ≥ 3 of pdFIX Concentrate and pure pdFIX. Instrument: KC-4

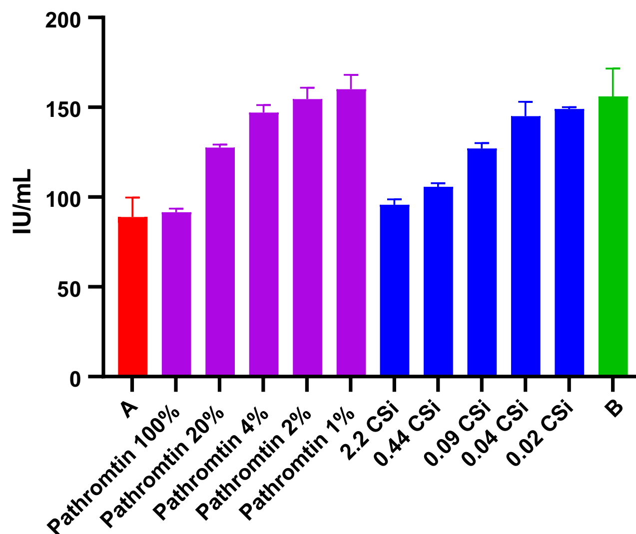

FIGURE 3 Assigned mean potencies of rFIX-FP in OS method with neat Pathromtin SL and mixtures of either Pathromtin SL with phospholipid-TGT (purple) or with 0.02-2.2 µL colloidal silica (CSi) included per mL phospholipid-TGT (blue). In all cases, coagulation was triggered by immediate (7-8 s) addition of FXIa/CaCl2 after adding the phospholipids in order to minimize any contact activation. Final assay concentrations of phospholipid and FXIa were 35 µmol/L and 1 IU/mL, respectively. Each bar represents the mean result from three determinations of two or three dilutions on KC-4. For comparison, pooled mean potencies, using data from Figures 1 and 2, with neat Pathromtin SL and 1.8 mL colloidal silica per L phospholipid-TGT and phospholipid-PF3 on KC-4 and STA-R Evolution, n = 15 (red bar, A) and SynthAFax, Rox Factor IX and phospholipid-TGT, phospholipid-PF3 with FXIa on KC-4, ACL TOP 500, STA-R Evolution and manual microplate method, n = 34 (green bar, B); are inserted

The results from in total seven analyses of the three FIX sources on ACL TOP and STA-R Evolution were all within one SD of the respective means.

Figure 3 shows that the assigned rFIX-FP potency decreased from 149 IU/mL to 96 IU/mL when the concentration of colloidal silica increased from 0.022 to 2.2 µL/L phospholipid emulsion. A similar decrease in rFIX-FP potency was obtained at increasing content of the silica-containing Pathromtin SL.

3.2 | Activation of FIX by tissue factor and FVIIa (extrinsic FIX activation)

rFIX-FP potency obtained by the tissue factor/FVIIa-based method was 71 ± 5 IU/mL (n = 3) vs 155 ± 12 IU/mL with the CS method.

4 | DISCUSSION

Regulatory authorities such as the European Pharmacopoeia requests the use of a one-stage clotting method for potency labelling of FIX therapeutics.[15] Labelling of rFIX-FP potency is made with the silica-based APTT reagent Pathromtin SL.[10]

The current study has aimed at identifying cause(s) of method discrepancies in potency estimation of rFIX-FP. Two sources of pdFIX were used for comparison.

The compiled results from Figures 1 and 2 strongly indicate that the use of silica as contact activator impairs activation of rFIX-FP as compared to activation of the plasma-derived IS FIX, resulting in lower assigned rFIX-FP potency. Specifically, about 90 IU/mL was obtained with colloidal silica incorporated with the two phospholipid sources as compared to 157 IU/mL when the same phospholipids were used in OS methods with FXIa included in the calcium chloride solution and hence omitting contact activation.

Importantly, these latter results were in line with those obtained with SynthAFax and the CS method.

For both pdFIX sources, there was a proper agreement of FIX potencies with all methods, including the two OS method variants with FXIa incorporated in the calcium chloride solution. Thus, there was no difference in activation of the two pdFIX samples vs activation of IS FIX. These results are in line with earlier published data, for example such as obtained in the calibration of the 5th IS FIX Concentrate, which demonstrated a very high agreement between OS and CS methods.[16]

Since similar results were obtained with the two phospholipid emulsions containing either 28 mol% or 8 mol% phosphatidylserine (the latter content corresponding closely to the amount of phosphatidylserine in platelet factor 3), the obtained results are not linked to a specific composition of phospholipids.

The effect of different silica concentrations was investigated in a modified OS method with silica provided either as pure substance or as the activator in Pathromtin SL (Figure 3). In both cases, use of the highest content of silica resulted in a rFIX-FP potency similar to the overall mean result with silica-based OS methods whereas gradual decrease of the silica concentration increased the assigned rFIX-FP potency, approaching the potency obtained in the absence of silica.

In these experiments, silica was added only 7-8 s before triggering coagulation by addition of FXIa and calcium chloride and any contact activation was therefore minimized. In this way, the direct effect of silica could be investigated.

The results clearly demonstrate that in the presence of silica, activation of rFIX-FP is impaired as compared to activation of IS FIX and hence deviates from the 'like-vs-like' principle.[16] It seems less probable that such a deviation occurs in the absence of a contact activator surface, hence implicating no overestimation of rFIX-FP on analysis with SynthAFax and the CS method.

An underestimation of rFIX-FP was also obtained when FIX activation was performed through the extrinsic pathway, that is by tissue factor-FVIIa, resulting in 71 IU/mL vs 155 IU/mL with the CS method. Independent support of an underestimation was also obtained by comparing FIXa generation at nominally identical FIX activities, resulting in a FIXa ratio of 0.76 for rFIX-FP vs the IS FIX and hence significantly below 1 (data not shown). Altogether, a plausible explanation of an impairment of rFIX-FP activation is steric hindrance by the fused albumin moiety in the interactions with silica and with the tissue factor-FVIIa complex.

Independent support for underestimation of rFIX-FP has also been obtained from extensive activation by FXIa during up to 60 minutes of pdFIX and rFIX-FP in a purified system with no FIX deficient plasma or phospholipids. The generated FIXa activities were correlated with FIX potencies and compared with results for the 5th IS FIX Concentrate. Whereas no differences vs the 5th IS were obtained for pdFIX, only rFIX-FP potencies assigned with SynthAFax and Rox Factor IX resulted in similar FIXa vs FIX ratios as obtained for the 5th IS.[12]

SynthAFax was the only ellagic acid-based APTT reagent used in our study. Ellagic acid-based reagents may, however, give deviating potency estimations.[10] Specifically, Actin FS causes underestimation of both N9-GP [3,6] and rFIX-FP [3,10] and is explained for N9-GP.[8] Hence, no general conclusions can be drawn regarding the performance of ellagic-based APTT reagents.

In this context, it should also be mentioned, as an example of APTT reagent diversities, that FXIa generation at the end of the contact activation phase may differ up to three-fold[12] and underestimation of FIX potency has been illustrated for the APTT reagent CK Prest, which, similar to Pathromtin SL, expresses a low level of generated FXIa.[12]

Modified rFVIII and rFIX products have introduced a challenge regarding potency assignments due to the discrepancies shown both within OS methods and vs CS methods. Guidelines have been published,[17-19] also recognizing 'real-life' difficulties in clinical laboratories. OS method discrepancies for EHL-rFIX products have resulted in an increased use of CS methods with due highlighting of discrepancies.

Activation of rFIX-FP results in removal of albumin and generation of FIXaβ, which is identical to native FIXaβ obtained after activation of pdFIX.[20] Thus, the molar specific activity should be the same in both cases. Our study demonstrates that silica impairs activation of rFIX-FP but not of pdFIX. Hence, rFIX-FP potency is underestimated in OS methods with silica-based APTT reagents including Pathromtin SL. In contrast, the SynthAFax-based OS method and CS methods appear to provide more reliable potency estimations. In clinical trials with rFIX-FP, demonstrating clinical efficacy, FIX activity was determined with Pathromtin SL.[21]

5 | CONCLUSION

Our study has addressed assay discrepancies on determination of FIX activity of rFIX-FP with one-stage and chromogenic methods.

The results demonstrate that use of silica as contact activator impairs activation of rFIX-FP as compared to activation of plasma-derived FIX such as a WHO International Reference Standard of FIX Concentrate. Similarly, impaired rFIX-FP activation was also obtained with FIX activation via the tissue factor-FVIIa complex.

Impaired activation of rFIX-FP results in an underestimation of rFIX-FP potency and may be caused by steric hindrance by the albumin moiety in the interaction with silica or with the tissue factor-FVIIa complex.

In contrast, about 80% higher rFIX-FP potency was obtained with the Rox Factor IX chromogenic method, an OS method with SynthAFax as well as variant OS methods, in which added FXIa replaces the contact activation phase and thus excludes surface interactions, which might cause assay discrepancies. Such methods quite probably provide more reliable FIX potency estimations of rFIX-FP.

In summary, the vulnerability of OS methods for FIX activity determination of EHL-rFIX´s, due to deviation from the 'like-vs-like' principle, is in sharp contrast to analysis of pdFIX sources.[16] Rather than advising against use of CS methods and an OS method with SynthAFax by referring to the discrepancy vs OS methods using

silica-based APTT reagents,[2,3] the results obtained in this study should encourage a careful review of the situation by concerned parties.

Finally, it should be emphasized that even though our results show that silica, such as included in Pathromtin SL, causes underestimation of rFIX-FP, they will have no implication on the dosing of the actual amount of rFIX-FP protein, since the clinical efficacy is well proven for the prescribed dosing.[21]

ADDENDUM

Steffen Rosén performed manual analyses on KC-4 and microplates and wrote the manuscript.

Pia Bryngelhed performed automated analyses on ACL TOP and STA-R Evolution, manual analyses in microplates and reviewed the manuscript.

ACKNOWLEDGEMENTS

Egon Persson (Cascade Consultant) and Per Rosén (Rossix AB) are gratefully acknowledged for critical review of the manuscript.

DISCLOSURE

Steffen Rosén is the founder of Rossix AB and currently performs consultancy for various companies including Rossix AB. Pia Bryngelhed is shareholder of Rossix AB.

ORCID

Steffen Rosén https://orcid.org/0000-0003-3452-0138

REFERENCES

1. Kitchen S, Gray E, Mertens K. Monitoring of modified factor VIII and IX products. *Haemophilia*. 2014;20(Suppl 4):36-42.
2. Kitchen S, Tiefenbacher S, Gosselin R. Factor activity assays for monitoring extended half-life FVIII and Factor IX replacement therapies. *Semin Thromb Haemost*. 2017;43:331-337.
3. Young GA, Perry DJ, International Prophylaxis Study Group (IPSG). Laboratory assay measurement of modified clotting factor concentrates: a review of the literature and recommendations for practice. *J Thromb Haemost*. 2019;17:1-7.
4. Sommer JM, Buyue Y, Bardan S, et al. Comparative field study: Impact of laboratory assay variability on the assessment of recombinant factor IX Fc fusion protein (rFIXFc) activity. *Thromb Haemost*. 2014;112:933-940.
5. Holm PK, Sörensen MH, Hermit MB, Ezban M. The activity of glycoPEGylated recombinant FIX (N9-GP) can be measured in two-stage chromogenic and one-stage clotting assays. *J Thromb Haemost* 2013;11(Suppl 2):828 (Abstract PB3:49-1).
6. Bowyer A, Hillarp A, Ezban M, Persson E, Kitchen S. Measuring factor IX activity of nonacog beta pegol with commercially available one-stage clotting and and chromogenic assay kits.: a two-centre study. *J Thromb Haemost*. 2016;14:1428-1435.
7. Rosén P, Rosén S, Ezban M, Persson E. Overestimation of N-glycoPEGylated factor IX activity in a one-stage factor IX clotting assay owing to silica-mediated premature conversion to activated factor IX. *J Thromb Haemost*. 2016;14:1420-1427.
8. Persson E, La Cour CC. Underestimation of N-glycoPEGylated factor IX one-stage clotting activity owing to contact activator-impaired activation. *Res Pract Thromb Haemost*. 2017;1:1-5.
9. St Ledger K, Feussner A, Kalina U, Jacobs I, Voigt C, Bensen-Kennedy D. Performance of a recombinant fusion protein linking coagulation factor IX with albumin (rFIX-FP) in the one-stage assay. *Haemophilia*. 2016;22(Suppl 4):60.
10. Horn C, Négrier C, Kalina U, Seifert W, Friedman KD. Performance of a recombinant fusion protein linking coagulation factor IX with recombinant albumin in one-stage clotting assays. *J Thromb Haemost*. 2019;17:138-148.
11. Tiebel O, Siegert G. Monitoring FIX prophylaxis and therapy with extended half-life factor IX concentrates in daily routine: Approaching the challenge by applying Rox Factor IX assay. *Hämostaseologie*. 2018;38(Suppl 4a). Abstracts GTH 2018: A35-A36 (Poster P024).
12. Rosén S, Bryngelhed P, Siegert G, Tiebel O. Underassignment of FIX potency of rFIX-Albumin fusion protein (rFIX-HSA) by one-stage methods demonstrated in studies on FXIa and FIXa generation. *Res Pract Thromb Haemost*. 2018;2(Suppl 1):117. (Abstract PB238).
13. Rosén S, Bryngelhed P. Recombinant Factor IX fused with albumin (rFIX-FP) is underassigned by one-stage methods with silica as contact activator. *Res Pract Thromb Haemost*. 2019;3(Suppl 1):197. https://doi.org/10.1002/rth2.12229.
14. Mikaelsson M, Oswaldsson U, Sandberg H. Influence of phospholipids on the assessment of factor VIII activity. *Haemophilia*. 1998;4(4):646-650.
15. Assay of human coagulation factor IX. European Pharmacopoeia; 2012: monograph 01/2008:20711.
16. Gray E, Hogwood J, Dougall T, Rigsby P. Collaborative study report on blood coagulation factor IX. WHO/BS/2015.2261 2015; 1-70.
17. Committee for Medicinal Products for Human Use (CHMP). Points to consider for assessors. New factor VIII and factor IX products: potency determination for labelling and assays for testing post-infusion samples. *Eur Med Agency*. 2015. EMA/CHMP/BPWP/231587:1–4.
18. Hubbard AR, Dodt J, Lee T, Mertens K, Seitz R, Srivastava A. Weinstein M on behalf of the Factor VIII and Factor IX Subcommittee of SSC/ISTH. Recommendations on the potency labelling of factor VIII and factor IX concentrates. *J Thromb Haemost*. 2013;11:988-989.
19. Dodt J, Hubbard AR, Wicks SJ, et al. Potency determination of factor VIII and factor IX for new product labelling and postinfusion testing: challenges for caregivers and regulators. *Haemophilia*. 2015;21(4):543-549.
20. Metzner HJ, Weimer T, Kronthaler U, Lang W, Schulte S. Genetic fusion to albumin improves the pharmacokinetic properties of factor IX. *Thromb Haemost*. 2009;102:634-644.
21. Kenet G, Chambost H, Male C, et al. Long-acting recombinant fusion protein linking coagulation factor IX with albumin (rFIX-FP) in children. Results of a phase 3 trial. *Thromb Haemost*. 2016;116:659-668.

How to cite this article: Rosén S, Bryngelhed P. FIX potency of rFIX-Albumin fusion protein is underestimated by one-stage methods using silica-based APTT reagents. *Haemophilia*. 2020;00:1–6. https://doi.org/10.1111/hae.13915