Journal of Thrombosis and Haemostasis, **17**: 567–573 DOI: 10.1111/jth.14394

REVIEW ARTICLE

Laboratory assay measurement of modified clotting factor concentrates: a review of the literature and recommendations for practice

GUY A. YOUNG,* DAVID J. PERRY† and FOR THE INTERNATIONAL PROPHYLAXIS STUDY GROUP (IPSG)
**Children's Hospital Los Angeles, University of Southern California Keck School of Medicine, Los Angeles, CA, USA; and †Cambridge Haemophilia and Thrombophilia Centre, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK*

To cite this article: Young GA, Perry DJ; for the 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**: 567–73.

Summary. Over the past several years, novel modified clotting factor concentrates (CFCs) have been introduced into practice and are now widely prescribed in the countries where they are licensed. These products allow for less frequent infusions of CFC, thereby providing improved convenience and/or higher trough levels. They have been extensively studied for prophylaxis, episodic treatment of bleeding and for surgical prophylaxis. One issue that has emerged regarding the clinical application of these products revolves around the measurement of infused CFC in the clinical coagulation laboratory. Recent studies have demonstrated significant problems with the measurement of correct FVIII/IX levels following infusion of novel CF VIII/IX concentrates. The source of this problem appears to be related to the tremendous variability of the APTT reagents that are used in the one-stage clotting assay, the most commonly used assay for determining factor levels. More specifically, the issue is related to the type of activator used in the reagents. Depending on the combination of the CFC and the APTT activator, the observed results may be either under- or overestimated to degrees that would be clinically relevant. Recommendations based on a review of published information regarding the potential for incorrect measurements of factor VIII/IX levels following infusion of recently developed, novel factor VIII/IX CFCs are presented for the clinician to use in clinical practice.

Keywords: extended half-life; factor concentrate; hemophilia; laboratory assays; reagents.

Correspondence: Guy A. Young, Children's Hospital Los Angeles, 4650 Sunset Blvd, Mail Stop 54, Los Angeles, CA 90254, USA
Tel.: +1 323 361 4141
E-mail: gyoung@chla.usc.edu

Received: 12 July 2018
Manuscript handled by: F. R. Rosendaal
Final decision: F. R. Rosendaal, 10 January 2019

Introduction

Hemophilia is an X-linked bleeding disorder that is manifested clinically by excessive bleeding, especially into joints and muscles, which eventually leads to hemophilic arthropathy, resulting in permanent joint damage [1]. Treatment for hemophilia consists of replacing the missing clotting factor (factor [F] VIII or FIX) on a regular basis (prophylaxis) or episodically (on-demand treatment). Clotting factor concentrate (CFC) therapy was developed over 50 years ago and during the following decades there has been a steady evolution of these essential replacement hemostatic products. Initially, clotting factor concentrates were plasma derived and contained other proteins (von Willebrand factor in the case of FVIII concentrates and factors II, VII and X in the case of FIX concentrates). Further refinements led to highly purified plasma-derived concentrates, followed by recombinant FVIII and IX products in the 1990s, which carry no risk of transmission of infectious agents, such as hepatitis C (HCV) or the human immunodeficiency virus (HIV). Shortcomings of CFCs are that they must be administered intravenously and, when given prophylactically, the frequency of infusions is two to four times per week. Over the past several years, several new factor products have been developed, which have been modified to extend their clearance from the circulation, allowing for equal efficacy yet with fewer infusions [2]. Although these extended half-life (EHL) products have been embraced by treaters and patients, the modifications to the CFC molecules have led to difficulties in measuring FVIII/IX activity with the one-stage clotting assay (OSCA), resulting in falsely decreased or increased FVIII/IX results [3]. Complicating the matter further is that there is no consistent pattern with respect to which EHL CFC and which class of reagents give increased or decreased results. As such, there is much confusion currently among hemophilia treaters and laboratory physicians/scientists regarding this problem. This manuscript is intended to concisely review what is currently known

about this new and very important clinical challenge, and to provide practical guidance as to how this potentially serious problem can be addressed.

Novel clotting factor concentrates

Recently, a new generation of CFCs termed EHLs have been developed, and several of these products are currently licensed for use in persons with hemophilia, whereas others are in advanced phases of clinical development [2]. Although there remains controversy over precisely which of the novel CFCs belong in the class of EHLs, for the sake of this manuscript, the following products will be discussed because they all are known to affect the results of the one-stage clotting assay (OSCA), the most commonly used factor assay in clinical practice: rFVIII Fc (Eloctate/Elocta, Bioverativ, Cambridge, MA, USA), rFVIII-PEG (Adynovate/Adynovi, Shire, Dublin, Ireland), N8-GP (Novo Nordisk, Copenhagen, Denmark), BAY-94-9027(Bayer, Berlin, Germany), rFVIII single chain (rFVIII-SC, Afstyla, CSL Behring, King of Prussia, PA, USA), rFIX Fc (Alprolix, Bioverativ), rFIX fusion protein (rFIX FP, Idelvion, CSL Behring) and N9-GP (Rebinyn, Novo Nordisk).

Factor assays

Currently there are two commercially available approaches for the measurement of factor VIII/IX levels, the OSCA mentioned above and the chromogenic assay (CHR) [4]. The final result of the OSCA is the formation of a clot, whereas the final result of the CHR assay is a color change in a chromogenic substrate, which is directly proportional to the amount of FXa generated.

The OSCA uses the activated partial thromboplastin time (APTT) as its base assay and is essentially a modified APTT mixing study using 50% patient plasma and 50% factor-deficient plasma of the factor that is being assayed. In addition to the factor-deficient plasma, the assay employs three components that make up the APTT reagents (an activator, phospholipids and calcium). Importantly, there are at least two dozen APTT reagents commercially available around the world, which vary in their activator and source of phospholipid [4]. The source of the phospholipid has not been proven to have any impact on the results of factor assays when used to measure the infused CFC for the new products listed earlier in this review, and thus will not be discussed further. The focus of discussion is the type of activator used as this has a major impact, as will be seen below, and therefore a basic understanding of what types of activators are available is critical. Among the commercially available APTT reagents, four different classes of activators are used: silica, ellagic acid, kaolin and polyphenolic acid, in order of frequency. Adding further to the confusion is that the types of silica and ellagic acid employed in the commercially available reagents vary, and these variations within the classes also exert different effects on the results of the factor assays. In other words, not all silica or ellagic acid reagents give the same results with the FVIII and FIX concentrates that are the subject of this review.

An alternative approach to the OSCA is the CHR assay [5]. There are several advantages to use of this assay as compared to the OSCA, as follows: (i) the OSCA relies on the patient's plasma to provide crucial components required for the final output of clot formation, whereas the CHR assay reagents remove this variable; and (ii) the CHR assay only requires FXa generation from the intrinsic tenase complex comprising factors VIIIa and IXa, thereby isolating the two clotting factors that are pathologically deficient in hemophilia. The CHR FVIII assay uses excess FIX in the assay, such that the amount of FIX in the patient's plasma is irrelevant, whereas the CHR FIX assay does the converse. Of note, there are different types of CHR assays, some with human proteins and some with bovine proteins, and in theory there could be differences between them with respect to measuring infused CFC, but this is unknown at this time.

Factor VIII concentrates and the OSCA

Table 1 summarizes published OSCA results according to concentrate and APTT reagent used. Interpretation of the data has several caveats. First, not all of the studies were performed in the same manner. Some were field studies that involved sending "spiked" samples of individual products to academic and commercial laboratories to get a "real world" sense of what issues were important. Other studies were limited to either one or just a few reference coagulation laboratories. The advantage of the field studies is that they provide a true sampling of what can be expected in clinical practice; however, they lack the detail and focus that more controlled studies are able to provide. For example, some field studies did not statistically test differences between reagents, but only simply reported whether a novel agent performed similarly to its comparator, a product whose characteristics are well established. This could lead to the false impression that there are no assay issues if, for example, half the reagents cause an overestimation of the results and the other half cause an equally significant underestimation. The more detailed studies carefully assessed different reagents with the respective CFC, which could demonstrate issues that may not be identified in field studies.

With this in mind, the major findings for the novel factor VIII concentrates are as follows.

1 Two products (rFVIII Fc and rFVIII-PEG) seemingly have no serious issues with any of the tested reagents; however, for both of these products, only field studies have been performed, with the aforementioned shortcomings [6]. Of note, for rFVIII-PEG, it was noted that

Table 1 Factor VIII assay results with the one-stage clotting assay (OSCA) according to concentrate and APTT reagent used, expressed qualitatively regarding whether the results will be correct, underestimated, overestimated or unknown

	rFVIII Fc[6]	rFVIII-PEG[7]	rFVIII-SC[10,11]	N8-GP[8]	BAY-94[9]
Silica reagents					
SynthaSIL	Correct	Correct	Underestimated	Correct	Correct
STA-PTT Automate	Correct	Correct	Underestimated	Underestimated	Underestimated
PTT-SP	Unknown	Unknown	Underestimated	Underestimated	Underestimated
Pathromtin SL	Correct	Correct	Underestimated	Correct	Correct
Triniclot Auto	Correct	Correct	Underestimated	Unknown	Unknown
Triniclot HS	Correct	Correct	Underestimated	Unknown	Unknown
Ellagic acid reagents					
Actin FS	Correct	Correct	Underestimated	Correct	Correct
Actin FSL	Correct	Correct	Underestimated	Correct	Correct
Synthafax	Unknown	Correct	Underestimated	Decreased	Correct
DG Synth	Unknown	Unknown	Unknown	Correct	Unknown
Kaolin reagents					
CK Prest	Correct	Correct	Underestimated	Correct	Unknown
Polyphenolic acid reagents					
Cephascreen	Correct	Correct	Underestimated	Correct	Correct

silica reagents led to somewhat lower FVIII levels than ellagic acid/polyphenolic acid reagents; however, these differences were not quantified [7].

2 Two other products (N8-GP and BAY-94-9027) have discrepancies with regard to silica reagents, with correct measurement with SythaSIL and Pathromtin SL but decreased recoveries with STA-PTT Automate and PTT-SP [8,9]. In addition, N8-GP had discrepant results with ellagic acid reagents, with correct measurements with Actin FS and DG Synth but decreased results with SynthaFax [8]. Leading to further confusion, this ellagic acid discrepancy was not seen with BAY-94-9027.

3 Finally, for the FVIII-SC molecule, all of the APTT reagents led to decreased recoveries [10,11]. The manufacturer has recommended that the OSCA can be used for assaying this agent so long as the result is multiplied by a factor of 2 and the Food and Drug Administration has allowed this recommendation to be included in the package insert for the USA [12]. The authors concur with previous comments from other authors [13] that this cannot be considered entirely safe, especially when an alternative approach is available (see below). The reason this is not considered safe is 2-fold. First, lot-to-lot variation in the same reagents may not yield the same results, and second, the degree of reduced recovery, while averaging approximately 50%, varied between approximately 30 and 70%. Therefore, for one reagent a recovery of 30% multiplied by 2 would give a "true" factor level of 60%, whereas a recovery of 70% multiplied by 2 would give a "true" factor level of 140%, so in some respects the multiplying factor could increase the difference between the true result and the observed result.

Factor VIII concentrates and the CHR assay

The data available regarding how well the CHR assay measures the recovery of novel CFCs is much more limited. However, what emerges from both field studies and smaller well-controlled studies is that there is far less variation with the CHR assay, and that measurements are more accurate and precise. Also, the SSR (sum of squared residuals) of FVIII CHR assays needed for single-dose PK is generally smaller than that by OSCA. There were two exceptions to this as follows. In a field study of rFVIIIFc, the CHR assay demonstrated an approximately 20% higher recovery when compared to a full-length rFVIII (Advate, Shire) at low, intermediate and normal levels of factor [6]. In addition, in a recent, small Swiss study, the CHR assay overestimated the recovery of rFVIII-PEG [14]. The remainder of the five novel FVIII concentrates considered in this review all demonstrated acceptable recoveries [3]. Therefore, the CHR assay can remove essentially all of the issues and confusion when compared to the OSCA and can be recommended to measure recoveries of all five of the novel FVIII concentrates. AS far as PK is concerned, it should be taken into account that the first part of the FVIII decay curve (FVIII > 25 IU dL^{-1}) is higher and steeper with respect to that determined by OSCA: sometimes, the half-life was shorter when FVIII was assayed by CHR than by OSCA [15].

Factor IX concentrates and the OSCA

Although it can be argued that two of the novel FVIII concentrates can be measured correctly by the OSCA, this cannot be stated for any of the rFIX concentrates: all have serious problems when it comes to measuring post-infusion FIX levels. Again, there is significant variation in the studies each manufacturer performed to address the assay issues and additional studies are warranted. With this in mind, the laboratory issues for the measurement of the three novel rFIX concentrates can be summarized as follows:

1 Two products (rFIX Fc and rFIX FP) can be measured correctly using OSCA with silica reagents, although not all silica reagents have been tested. Both, however, resulted in decreased recoveries with kaolin reagents [16,17]. With respect to (unspecified) ellagic acid reagents, rFIX Fc provides correct measurements at near-normal factor levels, but as levels drop to 0.2 IU mL^{-1} and 0.05 IU mL^{-1}, significant overestimations occur or are observed [16]. In contrast, and adding further confusion, Actin FS (an ellagic acid reagent) leads to significant underestimation of recovered factor after infusion with rFIX FP [17].
2 In contrast, N9-GP results in very significant (~4-fold) overestimation of FIX levels when certain silica reagents are used [18,19]. Interestingly, the mechanism of action for this has been explained in a well-designed study and is a result of massive activation of FIX by the PEG in the N9-GP molecule during the activation phase [19]. Other reagents (Actin FS [ellagic acid], SyntaSIL[silica] and STA-CK Prest [kaolin]) are known to underestimate the effects of N9-GP and the mechanism of action has been ascertained for some of these [20].This agent can be correctly measured with a rarely used polyphenolic acid reagent (Cephascreen) [21] and one ellagic acid reagent (SynthaFax) that is currently not available in the USA; another common ellagic acid reagent (Actin FS) results in decreased recoveries [18]. This agent can be measured correctly by kaolin and polyphenolic acid reagents; however, these are the least commonly used APTT reagents [18].

In summary, all three of these novel EHL FIX concentrates can be associated with incorrect FIX results if monitored with commonly used OSCA activators, and importantly, there is no consistency in results from one EHL FIX product to another.

Factor IX concentrates and the CHR assay

There are two currently available FIX chromogenic assays (Biophen Chromogenic factor IX, Biophen, Neuville, France, and the Rox FIX, Rossix, Mölndal, Sweden). There are very limited data on the performance of these assays; however, N9-GP can be correctly measured by both CHR assays [18], whereas rFIX Fc can be measured correctly by the Biophen assay [16]. Currently, data regarding the performance of these CHR assays with rFIX FP are not available.

Practical considerations

Availability of the OSCA and the CHR

The OSCA is by far the assay most coagulation laboratories in the world use for the measurement of factor levels, although in some parts of the world (Europe), CHR assays, especially for FVIII, are more commonly available. Compounding this issue is the fact that FIX CHR assays are not licensed in many parts of the world, including the USA. Moving forward, it is important that both FVIII CHR and FIX CHR assays are made more widely available in coagulation laboratories and licensed for use globally, as has been recommended by the National Hemophilia Foundation (of the USA) [22]. Furthermore, although it is highly unlikely that numerous hemophilia treatment centers will be able to add CHR assays to their laboratory menu for a variety of reasons, it will be important for at least some laboratories in each country to make these assays available to hemophilia patients by serving as a reference laboratory. Even if they are not licensed for clinical use, they can be made available as what are referred to as laboratory developed tests with a disclaimer.

Coagulation laboratory considerations

Most coagulation laboratories will use only one APTT activator for all APTT-based assays and the choice of activator often has to do with which instrument a laboratory uses and the contracts it has with the coagulometer manufacturer and whether the laboratory is more concerned about detecting low-level lupus anticoagulants or factor deficiencies, because one type of activator would typically be more effective for one and less effective for the other. For practical reasons, it is unlikely that laboratories will offer OSCA with different activators. It would be costly, complex to implement and have a high potential for errors. Furthermore, laboratories and clinicians would need to have a high degree of cooperation to ensure that the "correct" activator is used for each CFC clinicians prescribe when a factor assay is performed. In addition, laboratories receiving such samples should know which EHL CFC product they are being asked to assay if it is a recovery sample so that they can select the reagent that would be best suited if they have options.

Awareness of laboratory issues

At a minimum, clinicians treating hemophilia patients must be aware of the data presented above and should familiarize themselves with Tables 1 and 2. Although in the past a factor level was taken for granted as being correct, this is no longer true for the novel FVIII and IX products discussed in this review and it is crucial that clinicians understand the implications of this new reality. Both under- and overestimation of FVIII/IX levels can potentially result in patient harm. Underestimation can at best result in the expensive overuse of CFC, as higher than necessary doses may be prescribed, and at worst can put patients at risk of thrombosis by overdosing CFC. Overestimation can result in under-dosing CFC, which can result in bleeding. As an example, consider a

hypothetical patient who is going to have major surgery and a factor level is obtained immediately preoperatively that yields a result of 100%, which is a 5-fold overestimate, meaning the true factor level is 20%. This issue can perpetuate itself intraoperatively and postoperatively, whereby the patient may suffer from severe bleeding complications. Thus, it is incumbent upon hemophilia treaters to be aware of these serious issues and for laboratory physicians to communicate the potential for such issues to health care personnel involved with management of persons with hemophilia treated with the new FVIII/IX concentrates discussed in this review.

Practical solutions

Given the above, what could the broad hemophilia community do to ensure that when factor levels are requested the results can either be trusted (if done correctly) or dismissed (if the wrong methods are used). The first step to protecting patients from harm in this new era of CFCs is to ensure up to date and widespread education of both the hemophilia clinical team and the various personnel in the coagulation laboratory involved in performing and reporting the results. The responsibility for this education falls to both academia and industry. Leaders in both the laboratory and clinical realms of hemophilia care should develop methods to make sure that this important new information is widely disseminated through both research papers and presentations at meetings.

The second step would be to ensure that the necessary laboratory tests are readily available for clinicians to order. As discussed earlier, it is highly unlikely that each hemophilia treatment center will be able to be fully self-sufficient when it comes to having the necessary assays available. It is incumbent upon the medical and laboratory leadership of each country to work with reference laboratories such that they will offer the menu of necessary assays. This entails working with regulatory agencies to license the assays and make them available regardless of cost to all patients. Practically speaking, this could include the following options: (i) offer CHR assays nationally and/or regionally for both FVIII and FIX; (ii) develop a network of laboratories that use different APTT reagents that could work together to offer factor assays that could correctly measure factor levels regardless of which CFC a patient is receiving; (iii) require or request regulators, industry and scientific societies to offer support to commercial or academic laboratories to set up factor assays using the "correct" activator for their product and product-specific standards for patients who are on their products, and (iv) set up a system for centralized testing by OCSA or CHR such that local laboratories can measure their results against a "correct" standard to determine if the local methods can be used for measuring levels with any specific CFC. Importantly, laboratories will need to develop a nomenclature that will define which factor assay is actually performed. For example, a laboratory that offers both the OSCA and the CHR assay will need to consider different names for the assays. In other words, merely reporting results as a "factor VIII activity" is not going to be sufficient.

The third and final step would be for leading organizations such as the Scientific and Standardization Subcommittee of the International Society on Thrombosis and Haemostasis (ISTH) to develop some form of standardization within this highly complex environment, and to make recommendations regarding, for example, how laboratories report factor assay results for both OSCA and CHR assays.

Table 2 Factor IX assay results with the one stage clotting assay (OSCA) according to concentrate and APTT reagent used, expressed qualitatively regarding whether the results will be correct, underestimated, overestimated or unknown

	rFIX Fc[16]	rFIX FP[17]	N9-GP[18–21]
Silica reagents			
SynthaSIL	Correct	Correct	Underestimated
STA-PTT Automate	Unknown	Correct	Overestimated
PTT-SP	Unknown	Unknown	Overestimated
Pathromtin SL	Correct	Correct	Overestimated
Triniclot Auto	Correct	Unknown	Overestimated
Triniclot HS	Correct	Correct	Overestimated
Ellagic acid reagents			
Actin FS	Correct at normal levels but too high at FIX levels of 5–20%	Underestimated	Underestimated
Actin FSL	Correct	Unknown	Underestimated
Synthafax	Correct	Unknown	Correct
DG Synth	Correct	Unknown	Correct
Kaolin reagents			
CK Prest	Underestimated	Underestimated	Underestimated
Polyphenolic acid reagents			
Cephascreen	Correct	Unknown	Correct

Conclusions

The advent of novel, modified CFCs has enabled patients to continue receiving highly effective and safe therapies while providing the potential for higher trough factor levels and a reduction in the interval between dosing, thereby reducing the treatment burden. With these excellent new products, however, comes a new problem: measuring the "true" level of infused CFC. This problem is particularly acute with the EHL FIX concentrates, where each of the licensed products can lead to misleading results (both overestimation and underestimation). With respect to the FVIII products, one product results in misleading results with all OSCA reagents tested (rFVIII-SC). Although the FDA allows a multiplication factor to compensate for this issue, that strategy is not generally recommended. Two other products for which extensive studies have been performed demonstrate problems with some reagents but not others (BAY-94 and N8-GP). Two other products (rFVIII Fc and rFVIII-PEG) seem to have no serious issues with measurement; however, they have not had the same level of scrutiny through rigorously conducted studies as BAY-94-9027 and N8-GP. In conclusion, the hemophilia community of patients, clinicians, laboratory physicians, regulators and industry all share the responsibility for ensuring an awareness of this problem and for developing and implementing solutions, including clinical trials or registries to determine how often and seriously this problem surfaces in the clinic, such that persons with hemophilia using these novel CFCs are dosed correctly and safely.

Addendum

G.A. Young conceived and wrote the manuscript. The rest of the authors reviewed and edited the manuscript.

Acknowledgements

The authors thank the members of the IPSG Executive (V. Blanchette [Chair, Canada], L. Aledort [USA] and R. Ljung [Sweden]) for their review of the manuscript prior to submission. This manuscript is a product of the Pharmacokinetic (PK) Expert Working Group of the International Prophylaxis Study Group (the IPSG). Members of the PK Expert Working Group include: M. Morfini (Co-Chair, Italy), G.A. Young (Co-Chair, USA), V. Blanchette (Canada), K. Fischer (the Netherlands), A. Iorio (Canada) and S. Rangarajan (UK). D.J. Perry (UK) was an invited expert consultant and L. Tiseo (Hospital for Sick Children, Toronto, Canada) is coordinator for the IPSG PK Expert Working Group.

Disclosure of Conflict of Interests

G.A. Young reports personal fees from CSL Behring, Novo Nordisk, Shire and Bioverativ, during the conduct of the study.

References

1 Peyvandi F, Gargiola I, Young G. The past and future of haemophilia: diagnosis, treatment, and its complications. *Lancet* 2016; **388**: 187–97.
2 Young G, Mahlangu J. Extended half-life clotting factor concentrates: results from published clinical trials. *Haemophilia* 2016; **22** (suppl. 5): 25–30.
3 Kitchen S, Tiefenbacher S, Gosselin R. Factor activity assays for monitoring extended half-life FVII and factor IX replacement therapies. *Semin Thromb Hemost* 2017; **43**: 331–7.
4 Kitchen S, Signer-Romero K, Key NS. Current laboratory practices in the diagnosis and management of haemophilia: a global assessment. *Haemophilia* 2015; **21**: 550–7.
5 Peyvandi F, Oldenburg J, Friedman KD. A critical appraisal of one-stage and chromogenic assays of factor VIII activity. *J Thromb Haemost* 2016; **14**: 248–61.
6 Sommer JM, Moore N, McGuffie-Valentine B, Bardan S, Buyue Y, Kamphaus GD, Konkle BA, Pierce GF. Comparative field study evaluating the activity of recombinant factor VII Fc fusion protein in plasma samples at clinical haemostasis laboaratories. *Haemophilia* 2014; **20**: 294–300.
7 Turecek PL, Romeder-Finger S, Apostol C, Bauer A, Crocker-Buque A, Burger DA, Schall R, Gritsch H. A world-wide field survey and field study in clinical haemostasis laboratories to evaluate FVIII: C activity assay variablity of ADYNOVATE and OBIZUR in comparison with ADVATE. *Haemophilia* 2016; **22**: 957–65.
8 Hillarp A, Bowyer A, Ezban M, Persson P, Kitchen S. Measuring FVIII activity of glycopegylated recombinant factor VIII, N8-GP, with commercially available one-stage clotting factor and chromogenic assay kits: a two-centre study. *Haemophilia* 2017; **23**: 458–65.
9 Gu JM, Ramsey P, Evans V, Tang L, Apeler H, Leong L, Murphy JE, Laux V, Myles T. Evaluation of the activated partial thromboplastin time assay for clinical monitoring of PEGylated recombinant factor VIII (BAY 94-9027) for haemophilia A. *Haemophilia* 2014; **20**: 593–600.
10 Horn C, Zollner S, Muller-Cohrs J, Metzner H. Potency determination of single-chain rFVIII concentrate. *Haemophilia* 2016; **22** (Suppl. 4): 20.
11 St. Ledger K, Feussner A, Kalina U, Horn C, Metzner HJ, Bensen-Kennedy D, Blackman N, Veldman A, Stowers A, Friedman KD. International comparative field study evaluating the assay performance of AFSTYLA in plasma samples at clinical hemostasis laboratories. *J Thromb Haemost* 2018; **16**: 555–64.
12 http://www.afstyla.com/prescribing-information (accessed August 18, 2017)
13 Bowyer A, Key N, Dalton D, Kitchen S, Makris M. The coagulation laboratory monitoring of Afsytla single-chain FVIII concentrate. *Haemophilia* 2017; **23**: e460–70.
14 Bulla O, Poncet A, Alberio L, Asmis LM, Gahler A, Graf L, Nagler M, Studt JD, Tsakiris DA, Fontana P. Impact of a product-specific reference standard for the measurement of a PEGylated rFVIII activity: the Swiss Multicentre Field Study. *Haemophilia* 2017; **23**: e335–9.
15 Morfini M, Cinotti S, Bellatreccia A, Paladino E, Gringeri A, Mannucci PM; ReFacto-AICES Study Group. A multicenter pharmacokinetic study of B-domain deleted recombinant factor VIII concentrate using different assays and standards. *J Thromb Haemost* 2003; **1**: 2283–9.
16 Sommer JM, Buyue Y, Bardan S, Peters RS, Jiang H, Kamphaus GD, Gray E, Pierce GF. Comparative field study: impact of laboratory assay variability on the assessment of recombinant factor IX Fc fusion protein (rFIXFc) activity. *Thromb Haemost* 2014; **112**: 932–40.

17 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 (rIX-FP) in the one-stage assay. *Haemophilia* 2016; **22** (Suppl. 4): 60.
18 Bowyer AE, Hillarp A, Ezban M, Persson P, Kitchen S. Measuring factor IX activity of nonacog beta pegol with commercially available one-stage clotting and chromogenic assay kits: a two-centre study. *Haemophilia* 2016; **14**: 1428–35.
19 Rosen P, Rosen S, Ezban M, Persson P. 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**: 1420–7.
20 Persson E, Christoffersen CL. Underestimation of N-glycoPEGylated factor IX one-stage clotting activity owing to the contact activator-impaired activation. *Res Pract Thromb Haemost* 2017; **1**: 259–63.
21 Tiefenbacher S, Bohra R, Amiral J, Bowyer A, Kitchen S, Lochu A, Rosen S, Ezban M. Qualification of a select one-stage activated partial thromboplastin time-based clotting assay and two chromogenic assays for the post-administration monitoring of nonacog beta pegol. *J Thromb Haemost* 2017; **15**: 1901–12.
22 https://www.hemophilia.org/sites/default/files/document/files/masac-228.pdf (accessed August 18, 2017).