Factor VIII and Factor IX Activity Measurements for Hemophilia Diagnosis and Related Treatments

Annette E. Bowyer, FIBMS, PhD[1] Robert C. Gosselin, CLS[2]

[1] Department of Coagulation, Royal Hallamshire Hospital, Sheffield, United Kingdom
[2] Hemostasis and Thrombosis Center, University of California, Davis Health System, Sacramento, California

Address for correspondence Robert C. Gosselin, CLS, Hemostasis and Thrombosis Center, University of California, Davis Health System, Sacramento, CA 95817 (e-mail: rcgosselin@outlook.com).

Semin Thromb Hemost 2023;49:609–620.

Abstract

Accurate measurement of clotting factors VIII (FVIII) or IX (FIX) is vital for comprehensive diagnosis and management of patients with hemophilia A or B. The one-stage activated partial thromboplastin time (aPTT)-based clotting assay is the most commonly used method worldwide for testing FVIII or FIX activities. Alternatively, FVIII and FIX chromogenic substrate assays, which assess the activation of factor X, are available in some specialized laboratories. The choice of reagent or methodology can strongly influence the resulting activity. Variation between one-stage FVIII or FIX activities has been reported in the measurement of some standard and extended half-life factor replacement therapies and gene therapy for hemophilia B using different aPTT reagents. Discrepancy between one-stage and chromogenic reagents has been demonstrated in some patients with mild hemophilia A or B, the measurement of some standard and extended half-life factor replacement therapies, and the transgene expression of hemophilia A and B patients who have received gene therapy. Finally, the measurement of bispecific antibody therapy in patients with hemophilia A has highlighted differences between chromogenic assays. It is imperative that hemostasis laboratories evaluate how suitable their routine assays are for the accurate measurement of the various hemophilia treatment therapies.

Keywords

- one-stage factor assays
- chromogenic factor assays
- factor VIII
- factor IX
- hemophilia
- gene therapy

The X-linked, hemostatic disorders of hemophilia are caused by the absence or reduction of clotting factor VIII (hemophilia A, HA) or factor IX (hemophilia B, HB), which can lead to uncontrolled bleeding. It is estimated that more than 300,000 people have hemophilia worldwide.[1] The lower the measurable factor VIII (FVIII:C) or factor IX (FIX:C) functional "coagulant" activity, the more significant the bleeding diathesis. HA and HB are classified into severe (FVIII:C or FIX:C <1 dL), moderate (FVIII:C or FIX:C 1–5 IU/dL), and mild (FVIII:C or FIX:C >5 to <40 IU/dL) disorders based on the level of clotting factor activity.[2] Patients with mild HA or HB have fewer bleeding problems than those with moderate or severe forms, often only requiring replacement factor therapy following significant trauma or postoperatively. Patients with moderate hemophilia may bleed following minor trauma, whereas severely affected patients may exhibit spontaneous bleeding, which can occur into joint spaces (hemarthroses). Bleeding in untreated severe

article published online
December 6, 2022

Issue Theme Laboratory Diagnostics for Thrombosis and Hemostasis Testing—Part II; Guest Editors: Robert C. Gosselin, CLS and Kristi J. Smock, MD

DOI https://doi.org/10.1055/s-0042-1758870.
ISSN 0094-6176.

Thieme Medical Publishers, Inc., 333 Seventh Avenue, 18th Floor, New York, NY 10001, USA

hemophilia patients is variable, with annualized bleed rates (ABR) ranging from 0 to more than 50.[3] Treatment of hemophilia may be episodic (on-demand) following a bleed, or prophylactic (to prevent future bleeds) via regular injections of factor concentrate. Prior to the last decade, the treatment of hemophilia was with standard half-life (SHL) plasma-derived (pd) or recombinant (r) FVIII or (r)FIX. More recently, modifications to rFVIII or rFIX molecules have extended the half-life of the products in the circulation by around 1.5-fold for FVIII[4–7] and up to 5-fold for FIX,[8–10] while novel rebalancing therapies and gene therapy have greatly expanded the treatment options for hemophilia.[11–13] The prophylactic dosage of clotting factor concentrates may be regulated by a specific regimen of standard doses or tailored to each individual patient following measurement of the peak level (the FVIII:C or FIX:C immediately after treatment) and trough level (the lowest FVIII:C or FIX:C immediately prior to the next dose). The hemostasis laboratory serves a critical role in the diagnosis and management of HA or HB by providing screening tests (prothrombin time [PT] and activated partial thromboplastin time [aPTT]), specific factor assays (e.g., factor VIII, F8), and/or inhibitor studies (e.g., Bethesda assay). Factor assays can be used for diagnostic purposes (e.g., identifying a congenital or acquired factor deficiency), monitoring purposes (assessing the pharmacokinetics of a factor replacement therapy), assessing product quality control (e.g., FVIII:C in cryoprecipitate), or to assess product potency of factor concentrates.

Historically, the problems associated with these assays in the diagnosis and management of hemophilia have been attributed to the variability of results between assays, usually secondary to test methodology, calibration, and reagent (including factor deficient material) sources.[14,15] Improvements in factor assay performance within and between laboratories have emerged with advancing technologies (automated analyzers), laboratory performance guidelines such as those from the British Committee for Standardization in Haematology [BCSH], and proficiency testing.[16]

While biases still exist between laboratories in FVIII and FIX performance, in-house performance of these tests is usually constrained by operational limitations. These are typically instrument related, such as differences in the lower limit of quantitation (LLOQ) and can impact on determining hemophilia severity.

Clinicians are often unaware of laboratory limitations, and most have a relatively naive knowledge of their laboratory performance in clotting factor or inhibitor assays. Replacement products for treating HA and HB that were human (sometimes porcine) derived provided clotting factor activities as expected when using traditional laboratory methods. The expectation that all replacement therapies could be reliably monitored by any reagent or methodology changed when a B-domain–deleted (BDD) rFVIII (ReFacto, Wyeth Pharmaceutical) was approved by the U.S. Food and Drug Administration (FDA) in 2000. It was demonstrated that a ReFacto-specific calibrator was required to obtain accurate results, regardless of method (one-stage clotting assay [OSA] or chromogenic substrate assays [CSA]).[17,18] Since that time, there has been a proliferation of new hemophilia treatment strategies, including modified (polyethylene glycol(PEG) ylated, albumin-fused, FC-fusion) extended half-life (EHL) replacement products or gene therapy (in lieu of factor replacement). Each of these has laboratory challenges in accurately measuring factor activity.[19,20]

FVIII and FIX Factor Assays: General Laboratory Considerations

Unless the equipment and related reagents are designated for a hemophilia treatment center, it is likely that instrumentation and related reagent selection for clotting factor assays will be predicated on modified PT and aPTT assay testing. With the understanding that FVIII and FIX testing may be used outside the scope of hemophilia assessment, there are certain expectations that should be considered when using these platforms outside the general-purpose use of PT/aPTT screening or drug-monitoring testing, including but not limited to the following:

- Variables associated with instrumentation, calibrator source, calibration type and LLOQ, aPTT reagent, factor-deficient plasma source, and sample diluent (►**Table 1**). Likely, many of these variables are default protocols embedded within an instrument testing menu. Modifications or alterations of these defaulted protocol(s), including alternative reagents or calibrators, may constitute an in-house or laboratory-developed test which may have regional regulatory requirements for validation prior to clinical use.
- The selection of whether to use OSA or CSA may be predicated on additional considerations or restrictions such as reagent contracts, instrumentation, accreditation, or regional regulatory requirements.
- Anticoagulant interferences including heparins, parenteral direct thrombin inhibitors (DTIs), and direct oral anticoagulants (DOACs) may interfere with accurate performance of either OSA or CSA methods, with possible underestimation of factor activity. Some CSA assays may be less affected than other assays due to heparin neutralizers in reagents and higher sample dilutions. Some drugs may mimic a factor inhibitor and exhibit assay nonparallelism.[21]
- Nonspecific inhibitors (e.g., lupus anticoagulant) or other drug effects (e.g., lipoglycopeptide antibiotics) may interfere with OSA testing, although the inhibitor effect may be diminished due to sample dilutions for OSA testing.[22–24] CSA assays may be less affected due to higher sample dilution.
- Porcine-derived products (Obizur, porcine rFVIII, BAX801) are reliably assessed using OSA methods; CSA methods are less reliable.[25–28]
- For animal samples, OSA may be the preferred (only) option, although animal factor levels may not be comparable to humans, and thus calibration alterations may be required.[29]

Table 1 General factor assay considerations

Automation	Most coagulation analyzers have the capacity to perform OSA factor assays, usually dedicated to a single source of factor-deficient plasma, calibrator, and controls with clot detection by optical or mechanical means. Few instruments are programmed for CSA factor methods. Alterations to the instrument protocol may be required when using alternative materials and may require additional validation as required by regional regulatory agencies. Analyzers may have a dual OSA platform to accurately assess low factor activity levels (e.g., <15 IU/dL). Instruments without CSA protocols would require programming to adapt the methodology into an automated platform. CSA methods may also require dual platforms, where the low factor activity measurements requiring longer incubation and read times. Automation provides automatic calibration services, calibration curve fitting, patient calculations, and some instruments provide parallelism check to indicate presence of inhibitor
Calibration	All OSA and CSA methods require a calibration to provide a quantitative measurement. Calibrators should be traceable to recognized standard from reputable organizations such as World Health Organization (WHO), National Institute for Biological Standards and Control (NIBSC), or International Society of Thrombosis and Haemostasis (ISTH), and have an assigned activity using either OSA or CSA method. OSA methods typically have 5–7 calibration points, whereas CSA methods may have less, but more than 2 calibration points. Calibration curves can be linear, log–log; linear–log, polynomial, and use of derivatives. Patient samples should be tested at least at three different dilutions for OSA, and single dilution for CSA method
Factor-deficient plasma	Factor-deficient plasma (DP) can be immunodepleted, chemical depleted, or from congenital deficiency sources (with or without VWF in FVIII DP) and must contain <1 IU/dL of factor. DP may be lyophilized or frozen plasma. Whether one source is better than the other is for debate, each with their own advantages and disadvantages (e.g., cost, stability, reuse or freezing, instrument compatible without transferring to secondary vials, VWF levels, etc.)
aPTT reagent	Most commercial aPTT reagents are designed to be sensitive to changes in FVIII and FIX levels. Activator content (silica, kaolin, ellagic acid, polyphenols) and phospholipid type and source (e.g., animal, plant, or synthetic) are confounders to responsiveness of factor testing. Reagent considerations are required when the EHL factor testing is being performed on patients treated with EHL replacement products. Note that use of OSA for QC purposes, especially on cryoprecipitate products, may require a higher predilution of the sample than required with patient samples
Diluent	Suitable diluents used for making calibrator and plasma sample diluents include saline or buffered solutions such as Owren's, Owren-Koller, HEPES, imidazole buffer, FVIII or FIX-deficient plasma, and others

Abbreviations: aPTT, activated partial thromboplastin time; CSA, chromogenic assay; EHL, extended half-life; HEPES, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid; OSA, one-stage assay; VWF, von Willebrand factor.

FVIII and FIX Measurements for Diagnosis

The separation of hemophilia into HA and HB was first made in 1952[30] following the development of the OSA and two-stage clotting assays.[31–33] A variation of the OSA has been in world-wide use since that time, whereas the two-stage clotting assay is now performed only in a handful of specialized laboratories. The OSA is a modification to the aPTT by dilution of test plasma and addition of plasma that is completely devoid of the clotting factor to be tested (i.e., either FVIII or FIX) for hemophilia diagnosis. This factor-deficient plasma has a prolonged aPTT and any FVIII or FIX present in the patient plasma will shorten (or "correct") the aPTT. The clotting times generated in the patient plasma are compared with those of a reference or calibrator plasma and the concentration determined via a calibration curve.[34] The OSA is a heterogeneous test, with numerous combinations of reagents and instrumentation used worldwide. General considerations for factor assays include the use of automation, calibration, factor-deficient plasma source, aPTT reagent source, number of plasma dilutions, and diluent (►**Table 1**). aPTT reagents contain a contact activator, with a variety of materials used, including ellagic acid, kaolin or silica derivatives, as well as a variety of animal or plant-sourced phospholipids including phosphatidylserine, phosphatidylcholine, phosphatidylinositol, phosphatidylethanolamine, or sphingomyelin.[35] The sensitivity of aPTT reagents to mild deficiencies of FVIII and FIX is inconsistent.[36–38] While it is recommended that a prolongation to the aPTT be present when FVIII, FIX, or FXI are less than 30 IU/dL,[39] it follows that some reagents may have a normal aPTT in the presence of mild HA or HB which may compromise diagnosis.

In the 1980s, a chromogenic substrate FVIII assay (CSA) was introduced.[40] This assay involves activation of the test FVIII, then, in combination with added FIXa, activates FX to FXa. The FXa generated cleaves a FXa-specific chromophore and the resultant change in color is measured via optical density (OD). The OD of the test plasma is compared with those of a reference or calibrator plasma and the concentration determined via a calibration curve. The higher the color generation, the greater the level of FVIII. There are several kits on the market, which vary in source of reagents (human, bovine, or a mix), phospholipid type and concentration, buffers, and incubation time.

The chromogenic FIX assay is a recent addition to the laboratory repertoire. The assay principle is also a generation of FXa, then measurement by cleavage of a specific chromogenic substrate. There are, at the time of writing, three chromogenic FIX assay kits available which vary in their constituents and assay conditions.[41–44]

FVIII and FIX Measurements: Discrepancies in Mild Hemophilia

The diagnosis of severe, moderate, and some mild HA and HB phenotypes can be made by either OSA or CSA. The results of the two methods are generally comparable. However, there are more than 20 mutations in *F8* which are linked to significant discrepancy between OSA and CSA or two-stage clotting assay.[31] Assay discrepancy was initially reported in 1983 in four patients from two families with a twofold or greater FVIII:C measured by OSA than two-stage clotting assay.[45] This lower two-stage clotting or CSA form of assay discrepancy has been widely reported in Europe and Australia[46–50] and is caused by point mutations in *F8* at the A1–A2, A2–A3, or A1–A3 domain interfaces. These have been reported to increase the rate of dissociation of A2 domain leading to premature inactivation of FVIII.[51–53] The CSA has a longer incubation time than OSA; so, the untimely inactivation of FVIII manifests as reduced CSA activity, whereas the OSA FVIII:C is less impacted. The reverse discrepancy with a lower OSA compared with CSA has also been described. A further 10 mutations in *F8* have been linked to this type of assay discrepancy but in particular one mutation, p.Tyr365Cys/Phe has been commonly described in the United Kingdom.[54–57] These mutations, along with p.Glu340Lys and p.Ile388Thr, are thought to induce a conformational change resulting in a delay to thrombin activation of FVIII at p.Arg391.[54,58,59] This causes a lower OSA since FVIII is not activated as quickly as wild-type FVIII, but the longer incubation time in the CSA results in higher or normal activity. The reasons for reverse discrepancy with other mutations that are not near this thrombin activation site are unclear.[50] Some HA patients with FVIII assay discrepancy have reduced, but still discordant, OSA and CSA; so, the diagnosis is not usually compromised. However, in those patients where one result is within normal limits and the second is reduced, there may be a delay to diagnosis if only one assay method is routinely performed. It is therefore important to measure both OSA and CSA in patients with suspected mild HA. The clinical bleeding phenotype generally coincides with the lowest value observed, but this is dependent on the mutation present.[53]

Assay discrepancy in HB is rarely described, but when described appears to compromise the severity with patients alternating between moderate and mild HB. One report described 14 of 15 patients with FIX: c.572G > A; p.Arg191His or FIX: c.571C > T; p.Arg191Cys amino acid changes in *F9*. The mean OSA was 0.02 IU/mL and that of CSA was 0.06 IU/mL and patients reported a mild bleeding phenotype.[60] A further study reported 2.5-fold higher results by OSA compared with CSA.[61] The aPTT reagent composition has also been demonstrated to affect the OSA in patients with mild HB.[62]

FVIII and FIX Measurements: Monitoring of Standard Half-Life FVIII and FIX

The monitoring of pdFVIII or FIX and most SHL recombinant products are not influenced by OSA reagents. The exception is the B-domain–depleted (BDD) rFVIII concentrate, ReFacto, and its successor ReFacto AF (moroctocog alfa, Pfizer[63]). FVIII:C levels of ReFacto AF measured by OSA calibrated with a reference plasma may be 20 to 50% lower than expected; however, this difference can be reduced by use of either a product-specific reference plasma (ReFacto laboratory standard, Pfizer) in a standard OSA or using a plasma reference plasma in a CSA.[18] The recovery of ReFacto AF may also depend on the constituents of the aPTT reagent since not all aPTT reagents demonstrate lower results by OSA.[64,65] Chromogenic FVIII assays also recover close to the expected FVIII:C with SHL products; however, some SHL rFIX products may be underestimated by chromogenic FIX assays.[66,67] The United Kingdom Haemophilia Centre Doctors' Organization (UKHDCO) has published guidance for the laboratory monitoring of FVIII and FIX concentrates.[68] A summary of potential differences can be viewed in ►**Table 2**.

FVIII and FIX Measurements: Monitoring Extended Half-Life FVIII and FIX

One of the disadvantages of treatment with SHL FVIII and FIX therapy is the length of time that the product remains active in the circulation; for FVIII, this is approximately 12 hours

Table 2 Reagent and methodological discrepancy in the measurement of FVIII and FIX molecules

Molecule	One-stage V chromogenic assay discrepancy reported	One-stage assay reagent differences	Chromogenic assay reagent differences	References
Native FVIII	Yes in some patients with mild HA	No	No	48,56
Pd FVIII	Yes	No	No	125
SHL FVIII	Yes with BDD	Yes with BDD	No	65,118,126
EHL FVIII	Yes	Yes	No	68,81,84,127
FVIII mimetics	Yes	Not suitable for use	Yes	100,102,128
Native FIX	Yes in some patients with mild HB	No	No data	60,62,129
Pd FIX	No	Yes but limited data	No	66
SHL FIX	Yes	Yes	Yes	66,90,130
EHL FIX	Yes	Yes	Yes	66,79,90

Abbreviations: BDD, B-domain deleted; EHL, extended half-life; HA, hemophilia A; HB, hemophilia B; Pd, plasma derived; SHL, standard half-life.

Table 3 Modified factor VIII replacement products[131–135]

Name	Manufacturer	Factor VIII modification	Half-life[a] (h)	Approval date
ELOCTATE/ELOCTA (rFVIII-Fc BDD)	Bioverativ/SOBI	Fusion to Fc domain of IgG1	13–20	FDA Jun 2014 EMA Nov 2015
AFSTYLA (CSL627)	CSL Behring	Single chain—PEGylated	10–14	FDA May 2016 EMA Nov 2015
ADYNOVATE/ADYNOVI (Bax 855)	Takeda (Shire)	20-kDa branched PEGylated	12–15	FDA Dec 2016 EMA Jan 2018
JIVI (BAY 94–9027)	Bayer	Site-specific 60-kDa PEGylated	17–21	FDA Aug 2018 EMA Nov 2018
ESPEROCT (N8-GP)	Novo Nordisk	40-kDa glycoPEGylated	10–14	FDA Feb 2019 EMA Apr 2019

Abbreviations: EMA, European Medicines Agency; FDA, Food and Drug Administration; KDa, kilodalton; PEG, polyethylene glycol.
[a]Half-life represents mean values, and is age dependent (pediatrics vs. adults)—source is prescribing information from each respective drug.

and for FIX approximately 20 hours.[69] There have been several approaches undertaken to extend the half-life of each protein including fusion with polyethylene glycol (PEG),[5,6,70–72] albumin,[73,74] or the Fc fragment of IgG.[75–77] This has resulted in commercially available EHL FVIII (►**Table 3**) and FIX (►**Table 4**) products for the treatment of HA and HB, respectively.

Even with liberal acceptability for factor recovery of 25 to 30%, it became apparent during clinical and laboratory field trials with some EHL products and some aPTT reagents that significant discrepancy in measured FVIII or FIX recovery could be reproducibly demonstrated. It cannot be assumed that EHL molecules that use the same modification technology (such as PEG) will exert the same effect on aPTT reagents that share the same activator and phospholipid source. Likewise, it cannot be assumed that aPTT reagents that share the same activator and phospholipid source will have the same response to those EHL with the same modification.[78,79] EHL prescribing information may assist clinicians for acceptable or recommended laboratory methods, but it is unlikely that many clinicians are aware of the FVIII or FIX method used in their laboratory and the limitations of those assays. Additionally, most physicians are usually unaware of their laboratory's performance in FVIII, FIX, or related inhibitor assays as assessed by External Quality Assurance programs required for comparing local laboratory performance to regional or international peers.

There are currently five FVIII EHL products available for clinical use (►**Table 3**). Esperoct (previously N8-GP, Novo Nordisk, Denmark) was underestimated by up to 50% by silica-activated aPTT SP,[80,81] while Jivi (previously Bay 94–9027-, Bayer AG, Germany) was underestimated by some silica-based aPTT reagents and overestimated by some kaolin-based aPTT reagents.[82] The single-chain recombinant FVIII, Afstyla (CSL Behring, Germany), is underestimated by approximately 45% by OSA,[83,84] and consequently the manufacturer recommends the CSA.[85] The manufacturer also states that if the OSA is used, then a correction factor of 2 can be applied to obtain the final result. This approach is not internationally recommended due to differences between one-stage assays.[68] The CSA has been reported to achieve acceptable recovery with all currently licensed EHL FVIII concentrates. Additional modifications to rFVIII, which combines BDD rFVIII with Xten polypeptides and a fragment of von Willebrand factor, have improved the half-life extension to more than threefold compared with SHL in clinical studies.[86]

There are currently three EHL FIX concentrates licensed for use in some countries. These have been modified by Fc fusion,[77] albumin fusion,[9] or glycopegylation[87] (►**Table 4**). Under or over estimation with aPTT reagents in the OSA has been reported for each product.[66,79,88–90] CSAs are also varied in their response.[66,91]

In 2020, there were three guidance publications that provided recommendations for or against FVIII and FIX OSA or CS assays for EHL products.[68,92,93] Of particular concern is (1) limited data for nondescribed aPTT reagents, (2) insufficient or discordant data obtained from clinical or

Table 4 Modified factor IX replacement products[136–138]

Name	Manufacturer	Factor VIII modification	Half-life[a] (h)	Approval date
ALPROLIX (rFIX-Fc)	Bioverativ/SOBI	Fusion to Fc domain of IgG1	68–94	FDA Mar 2014 EMA May 2016
IDELVION (rIX-FS CSL654)	CSL Behring	Fusion to albumin	~90	FDA Mar 2016 EMA May 2016
REBINYN/REFIXIA (N9 = GP)	Novo Nordisk	40-kDa glycoPEGylated	70–89	FDA May 2017 EMA Mar 2017

Abbreviations: EMA, European Medicines Agency; FDA, Food and Drug Administration; kDa, kilodalton; PEG, polyethylene glycol.
[a]Single-dose, half-life mean values, and is age dependent (pediatrics vs. adults)—source is prescribing information from each respective drug.

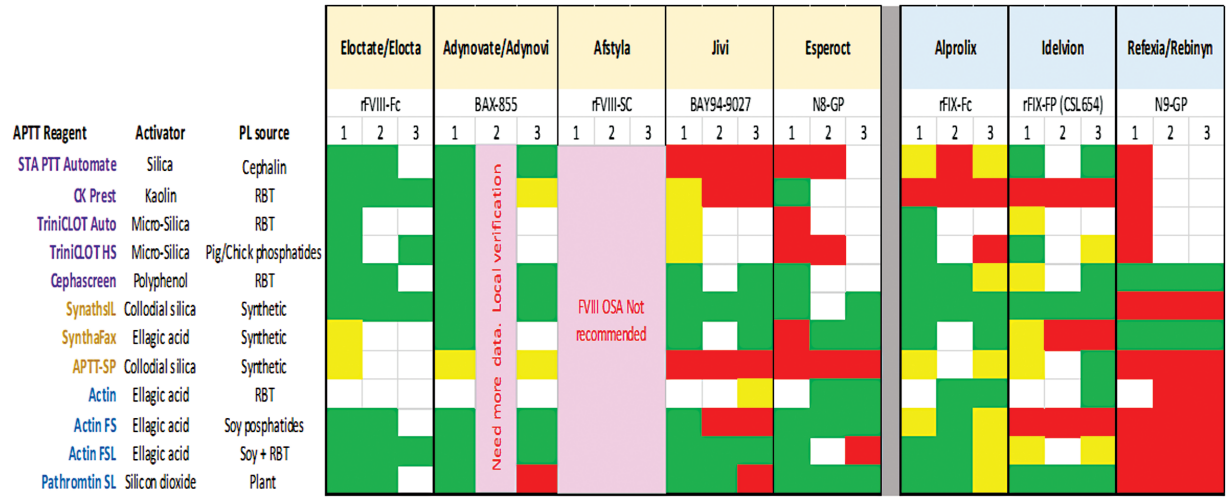

Fig. 1 Recommended or rejected OSA methods for measuring FVIII and FIX EHL products from recent publications.[68,92,93] Columns labeled "1" reflect recommendations from Peyvandi et al,[92] columns labeled "2" from Gray et al,[68] and columns labeled "3" from Jeanpierre et al.[93] Green cells indicate a recommended method, yellow cells indicate insufficient or conflicting clinical or field trial data, and red cells indicate rejected method. A white cell indicates this method was not specifically addressed by authors. *Note*: Afstyla can only be measured using a suitable FVIII chromogenic assay. *Note*: For Adynovate/Adynovi, the Kihlberg et al[60] group could not recommend any OSA method. PL, phospholipid; RBT, rabbit brain thromboplastin.

field studies, and (3) discordant OSA or CSA recommendations between these publications. It is likely that a clinical laboratory will provide a single OSA method for both FVIII and FIX, usually due to contractual agreements or instrument default methods. Therefore, when evaluating the three guidance documents, it is necessary to recommend which OSA method is suitable for monitoring FVIII and FIX EHL concentrates (►**Fig. 1**). For CSA methods, there was mostly concordance between these guidance recommendations, with notable exception for Esperoct and Idelvion (►**Fig. 2**).

It is imperative that laboratories assess their local assays for suitability to measure EHL FVIII and FIX concentrates that may be used to treat their patients. For those aPTT methods not adequately described or recommended, implementing a reagent for EHL based on other reagent platforms with similar activator may not be a suitable means for predicting suitability for EHL monitoring (►**Fig. 3**). Given the variability within a given reagent platform (►**Figs. 1** and **2**), it is likely that more than one FVIII or FIX method may be required to accurately monitor all EHL replacement therapies.

FVIII Measurements: FVIII Mimetics

FVIII mimetics, including emicizumab (Hemlibra, Roche Chugai) and Mim8 (Novo Nordisk), are humanized bispecific antibodies directed to human FIX (FIXa) and FX, which

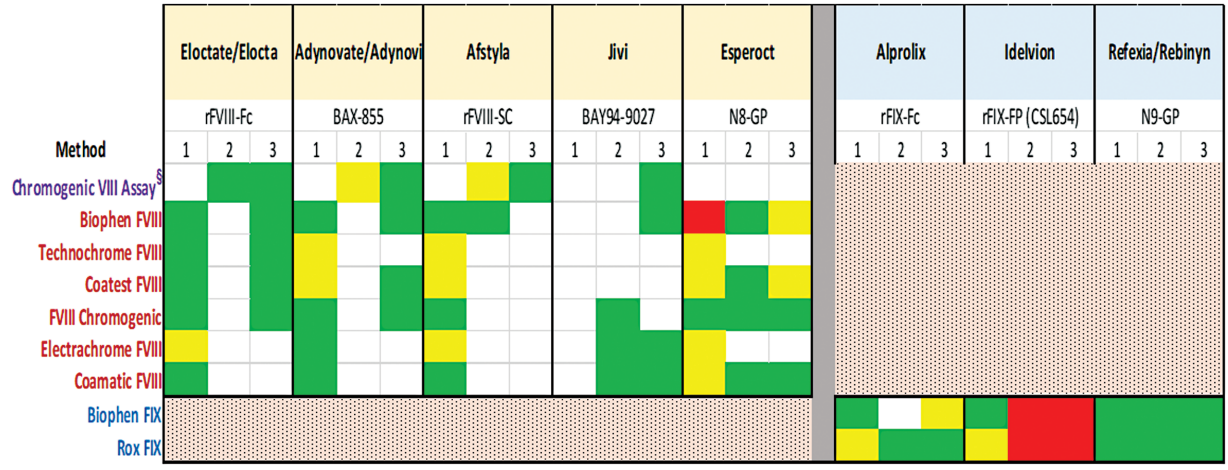

Fig. 2 Recommended or rejected CS methods for measuring FVIII and FIX EHL products from recent publications.[68,92,93] Columns labeled "1" reflect recommendations from Peyvandi et al,[92] columns labeled "2" from Gray et al,[68] and columns labeled "3" from Jeanpierre et al.[93] Green cells indicate a recommended method, yellow cells indicate insufficient or conflicting clinical or field trial data, and red cells indicate rejected method. A white cell indicates this method was not specifically addressed by authors. §Generic recommendations for CS VIII methods, no specific reagent(s) may be noted. *Note*: Kihlberg et al[60] group did not provide recommendations for Adynovate/Adynovi based on limited clinical or field trial data.

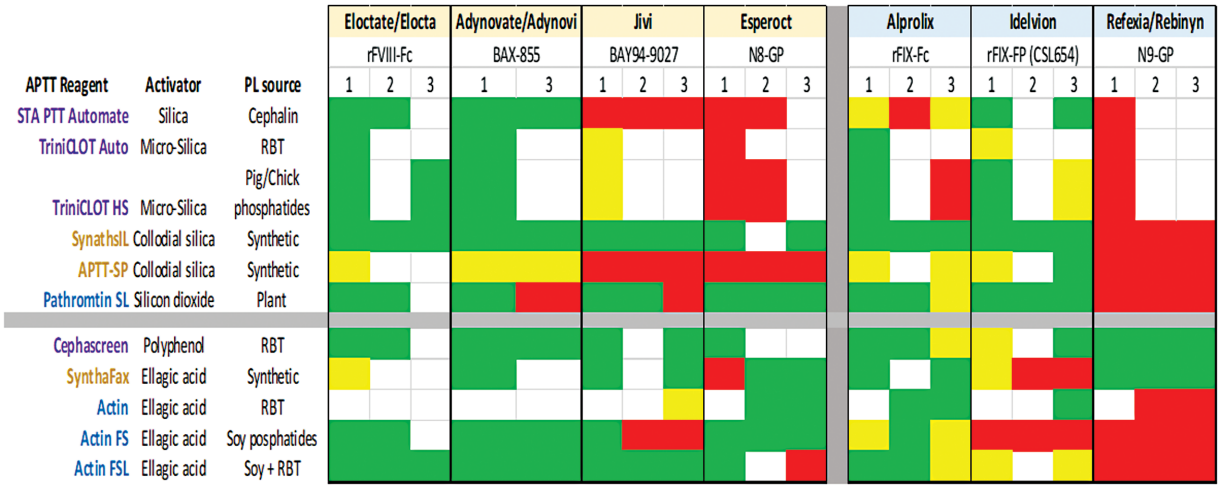

Fig. 3 Aligning aPTT reagents by activators (silica or polyphenols) of the recommended or rejected CS methods for measuring FVIII and FIX EHL products from recent publications.[68,92,93] Columns labeled "1" reflect recommendations from Peyvandi et al,[92] columns labeled "2" from Gray et al,[68] and columns labeled "3" from Jeanpierre et al.[93] Green cells indicate a recommended method, yellow cells indicate insufficient or conflicting clinical or field trial data, and red cells indicate rejected method. A white cell indicates this method was not specifically addressed by authors. PL, phospholipid; RBT, rabbit brain thromboplastin.

activate FX in the absence of FVIIIa.[94,95] Emicizumab is licensed for use in Europe, Australia, and the United States, in patients with HA and anti-FVIII antibodies and severe HA without antibodies.[96] Bispecific antibodies do not require preactivation to be functional, unlike FVIII; thus, the action on FX is more rapid[97] and this impacts on some hemostasis tests.[98–100] The aPTT dramatically shortens, even at subtherapeutic concentrations and any aPTT-based assays will also be affected, including OSA for FVIII.[98,101] OSA FVIII is artificially increased, often far above the top of the reference range. Several organizations have published guidance for the monitoring of patients receiving emicizumab therapy.[102,103] Quantitative measurement of the emicizumab drug concentration can be made by modifying the OSA to use an emicizumab-specific calibrator and high plasma dilutions. The use of CSA which use bovine FX can measure endogenous FVIII due to their insensitivity to emicizumab at therapeutic concentrations.[104] The monitoring for FVIII inhibitors in patients receiving these products create an additional challenge, with modification to existing Bethesda or Nijmegen methods having been described.[105]

FVIII and FIX Measurements: Rebalancing Therapies

New classes of drugs, not based on the replacement of FVIII or IX molecules, are currently in clinical trials for prophylaxis of hemophilia patients. They include the small interference RNA (siRNA) antithrombin knockdown therapy (fitusiran, Sanofi),[106] anti–tissue factor pathway inhibitor (TFPI) monoclonal antibodies (concizumab, Novo Nordisk, and marstacimab, Pfizer),[107,108] and serpins against activated protein C.[109] These drugs are able to increase thrombin generation in the absence of FVIII and FIX, although it is unlikely that monitoring these rebalancing therapies using routine hemostasis assays will be clinically useful, or easily applied.

FVIII and FIX Measurements: Gene Therapy

Gene therapy trials have been running for FVIII and FIX for several years, with numerous currently active and recruiting clinical trials for both HA and HB.[12,110–112] Recent guidance from the U.S. FDA for human gene therapy in hemophilia has a section detailing the necessity for laboratory testing to include both chromogenic and one-stage assays with a variety of reagents in vitro and a comparative field study in patient plasma.[113]

Differences in FVIII or FIX transgene expression measured by OSA and CSA have been reported in hemophilia patients who have received certain gene therapy products (►**Table 5**). In HA, an approximate 1.6-fold higher FVIII:C OSA FVIII:C than CSA has been reported.[114–117] Curiously, this is the reverse of the pattern observed in some BDD-rFVIII molecules.[17,118] Rosen et al proposed that the higher OSA compared with CSA in AAV5-FVIII-SQ molecules is caused by accelerated early FXa and thrombin generation, and shorter clotting times generate higher reported FVIII activity levels. This trend was recorded in transgene FVIII:C and the rFVIII-SQ molecule.[119] Kinetic assays highlighted the assay differences, but the underlying mechanism is currently unknown.

There has been a difference in recovery of FVIII:C with a limited number of different aPTT reagents in the clinical trials. Ideally, laboratory field studies would provide performance characteristics for OSA or CS methods not used in the clinical trials.[119]

Discrepancy between OSA and CSA FIX:C has also been reported following FIX gene therapy for HB. Gene therapy approaches that use FIX-Padua, a naturally occurring mutation with higher FIX:C than wild-type FIX,[120] have observed differences in one-stage FIX:C measured using a variety of aPTT reagents and also between OSA and CSA in both the transgene FIX expressed by patients and plasma spiked with the FIX molecule.[121–124] The degree of OSA *versus* CSA discrepancy may be up to twofold; again, OSA activities

Table 5 Selected OSA methods from clinical trials for FVIII and FIX gene therapy

	OSA/CA (slope) Hemophilia A[119]	OSA/CA (ratio) Hemophilia B[121]	OSA/CA (slope) Hemophilia B[a]
Actin FS	1.29–1.88	ND	2.03
Actin FSL	1.52–1.66	1.2–1.6	1.10
SynthasIL	1.53–2.01	1.3–2.4	1.38
Triniclot aPTT HS	1.67	ND	ND
CK Prest	ND	0.7–2.2	ND
PTT-Automate	ND	1.0–2.8	ND
Pathromtin SL	ND	ND	1.49

Abbreviations: CA, chromogenic assay; ND, no data; OSA, one-stage clotting assay.
[a]Author (RCG) unpublished data.

are higher than CSA, but the degree of discrepancy between OSA and CSA seems to vary between patients.[121]

With noted observations of differences between OSA and CS methods in clinical studies, the primary endpoint in hemophilia gene therapy is the phenotype of the patient. It is unclear whether routine monitoring is required for gene therapy but is likely necessary for patients who require surgical intervention. It is unknown whether additional factor replacement therapies would be required in gene therapy patients with acute bleeding events, which may add an additional confounder to interpreting OSA or CS results. Hemostasis laboratories may be required to establish unfamiliar assays to facilitate consistent management of gene therapy patients within their center and for those who travel between centers.

Conclusions

For decades, the clinical laboratory has provided the necessary testing for the diagnosis and management of patients with hemophilia. The OSA FVIII and FIX methods are still the traditional workhorses in assessing these patients, with improvements in testing accuracy and precision resulting from technological advances, guidance documents, and robust external proficiency assessment programs. However, the diagnostic limitations of OSA in certain gene mutations and the OSA challenges associated with monitoring modified factor replacement or gene therapies require clinical laboratories to consider secondary options for FVIII and FIX testing to aide clinicians who manage hemophilia patients. A multidisciplinary approach between clinical and laboratory teams is necessary to provide optimum diagnosis and monitoring of treatment for patients with hemophilia. A combination of OSA and chromogenic assay for both FVIII and FIX would appear to be the most favorable test combination to address the current diagnostic and monitoring challenges in hemophilia patients.

Conflict of Interest

A.E.B. reports grants and personal fees from Novo Nordisk, personal fees from Stago, personal fees from Takeda, grants and personal fees from Roche, personal fees from Sobi, and personal fees from Pfizer, outside the submitted work.

R.C.G. reports personal fees from Sysmex America Incorporated and from Diagnostica Grifols, and honoraria from Diagnostica Stago, outside the submitted work.

References

1 Srivastava A, Brewer AK, Mauser-Bunschoten EP, et al; Treatment Guidelines Working Group on Behalf of The World Federation Of Hemophilia. Guidelines for the management of hemophilia. Haemophilia 2013;19(01):e1–e47
2 White GC II, Rosendaal F, Aledort LM, Lusher JM, Rothschild C, Ingerslev JFactor VIII and Factor IX Subcommittee. Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on Thrombosis and Haemostasis. Thromb Haemost 2001;85(03):560
3 Aledort LM, Haschmeyer RH, Pettersson HThe Orthopaedic Outcome Study Group. A longitudinal study of orthopaedic outcomes for severe factor-VIII-deficient haemophiliacs. J Intern Med 1994;236(04):391–399
4 Tiede A, Brand B, Fischer R, et al. Enhancing the pharmacokinetic properties of recombinant factor VIII: first-in-human trial of glycoPEGylated recombinant factor VIII in patients with hemophilia A. J Thromb Haemost 2013;11(04):670–678
5 Coyle TE, Reding MT, Lin JC, Michaels LA, Shah A, Powell J. Phase I study of BAY 94-9027, a PEGylated B-domain-deleted recombinant factor VIII with an extended half-life, in subjects with hemophilia A. J Thromb Haemost 2014;12(04):488–496
6 Turecek PL, Bossard MJ, Graninger M, et al. BAX 855, a PEGylated rFVIII product with prolonged half-life. Development, functional and structural characterisation. Hamostaseologie 2012;32 (Suppl 1):S29–S38
7 Mahlangu J, Powell JS, Ragni MV, et al; A-LONG Investigators. Phase 3 study of recombinant factor VIII Fc fusion protein in severe hemophilia A. Blood 2014;123(03):317–325
8 Powell JS, Pasi KJ, Ragni MV, et al; B-LONG Investigators. Phase 3 study of recombinant factor IX Fc fusion protein in hemophilia B. N Engl J Med 2013;369(24):2313–2323
9 Santagostino E, Martinowitz U, Lissitchkov T, et al; PROLONG-9FP Investigators Study Group. Long-acting recombinant coagulation factor IX albumin fusion protein (rIX-FP) in hemophilia B: results of a phase 3 trial. Blood 2016;127(14):1761–1769
10 Collins PW, Young G, Knobe K, et al; Paradigm 2 Investigators. Recombinant long-acting glycoPEGylated factor IX in hemophilia B: a multinational randomized phase 3 trial. Blood 2014;124 (26):3880–3886

11 Pipe SW, Ragni MV, Negrier C, et al. Fitusiran, an RNAi therapeutic targeting antithrombin to restore hemostatic balance in patients with hemophilia A or B with or without inhibitors: management of acute bleeding events. Blood 2019;134:1138
12 Batty P, Lillicrap D. Gene therapy for hemophilia: current status and laboratory consequences. Int J Lab Hematol 2021;43(Suppl 1):117–123
13 Mahlangu J, Oldenburg J, Paz-Priel I, et al. Emicizumab prophylaxis in patients who have haemophilia A without inhibitors. N Engl J Med 2018;379(09):811–822
14 Christensen RL, Triplett DA. Factor assay (VIII and IX) results in the College of American Pathologists Survey Program (1980-1982). Am J Clin Pathol 1983;80(4, Suppl):633–642
15 Arkin CF, Bovill EG, Brandt JT, Rock WA, Triplett DA. Factors affecting the performance of factor VIII coagulant activity assays. Results of proficiency surveys of the College of American Pathologists. Arch Pathol Lab Med 1992;116(09):908–915
16 Wasi S, Murray SA, Gill P. Proficiency testing of factor VIII:C activity assays at the Canadian Red Cross Society National Reference Laboratory. Vox Sang 1994;67(01):1–7
17 Ingerslev J, Jankowski MA, Weston SB, Charles LAReFacto Field Study Participants. Collaborative field study on the utility of a BDD factor VIII concentrate standard in the estimation of BDDr Factor VIII:C activity in hemophilic plasma using one-stage clotting assays. J Thromb Haemost 2004;2(04):623–628
18 Pouplard C, Caron C, Aillaud MF, et al. The use of the new ReFacto AF Laboratory Standard allows reliable measurement of FVIII:C levels in ReFacto AF mock plasma samples by a one-stage clotting assay. Haemophilia 2011;17(05):e958–e962
19 Kitchen S, Tiefenbacher S, Gosselin R. Factor activity assays for monitoring extended half-life FVIII and factor IX replacement therapies. Semin Thromb Hemost 2017;43(03):331–337
20 Weyand AC, Pipe SW. New therapies for hemophilia. Blood 2019;133(05):389–398
21 Adcock DM, Strandberg K, Shima M, Marlar RA. Advantages, disadvantages and optimization of one-stage and chromogenic factor activity assays in haemophilia A and B. Int J Lab Hematol 2018;40(06):621–629
22 Brandt JT, Triplett DA, Rock WA, Bovill EG, Arkin CF. Effect of lupus anticoagulants on the activated partial thromboplastin time. Results of the College of American Pathologists survey program. Arch Pathol Lab Med 1991;115(02):109–114
23 Gosselin RC, King JH, Janatpur KA, Dager WH, Larkin EC, Owings JT. Effects of pentasaccharide (fondaparinux) and direct thrombin inhibitors on coagulation testing. Arch Pathol Lab Med 2004;128(10):1142–1145
24 Exner T, Rigano J, Favaloro EJ. The effect of DOACs on laboratory tests and their removal by activated carbon to limit interference in functional assays. Int J Lab Hematol 2020;42(Suppl 1):41–48
25 Bowyer A, Gray E, Lowe A, et al. Laboratory coagulation tests and recombinant porcine factor VIII: A United Kingdom Haemophilia Centre Doctors' Organisation guideline. Haemophilia 2022;28(03):515–519
26 EMA Accessed September 30, 2022; 04/03/2020 at: https://www.ema.europa.eu/en/documents/product-information/obizur-epar-product-information_en.pdf2015
27 Obizur US prescribing information. Baxalta US Inc. Lexington, MA FPI-0207 Revised 09/2021. Accessed 16 November 2022 at: https://www.shirecontent.com/PI/PDFs/OBIZUR_USA_ENG.pdf
28 Shima M, Lillicrap D, Kruse-Jarres R. Alternative therapies for the management of inhibitors. Haemophilia 2016;22(Suppl 5):36–41
29 Karges HE, Funk KA, Ronneberger H. Activity of coagulation and fibrinolysis parameters in animals. Arzneimittelforschung 1994;44(06):793–797
30 Biggs R, Douglas AS, MacFarlane RG, Dacie JV, Pitney WR, Merskey. Christmas disease: a condition previously mistaken for haemophilia. BMJ 1952;2(4799):1378–1382
31 Barrowcliffe TW. Methodology of the two-stage assay of factor VIII (VIII:C). Scand J Haematol Suppl 1984;41(Suppl 41):25–38
32 Denson KWE, Wilkins T. Semi-automation of the two-stage factor VIII assay. Clin Lab Haematol 1980;2:311–316
33 Langdell RD, Wagner RH, Brinkhous KM. Effect of antihaemophilic factor on one-stage clotting tests. A presumptive assay for haemophilia and a simple antihaemophilic factor assay procedure. J Lab Clin Med 1953;41(04):637–647
34 Mackie I, Cooper P, Lawrie A, Kitchen S, Gray E, Laffan MBritish Committee for Standards in Haematology. Guidelines on the laboratory aspects of assays used in haemostasis and thrombosis. Int J Lab Hematol 2013;35(01):1–13
35 Kitchen S, Cartwright I, Woods TAL, Jennings I, Preston FE. Lipid composition of seven APTT reagents in relation to heparin sensitivity. Br J Haematol 1999;106(03):801–808
36 Bowyer A, Kitchen S, Makris M. The responsiveness of different APTT reagents to mild factor VIII, IX and XI deficiencies. Int J Lab Hematol 2011;33(02):154–158
37 Toulon P, Eloit Y, Smahi M, et al. In vitro sensitivity of different activated partial thromboplastin time reagents to mild clotting factor deficiencies. Int J Lab Hematol 2016;38(04):389–396
38 Bowyer AE. The sensitivity of two new APTT reagents to factors VIII, IX and XI. [abstract]Res Pract Thromb Haemost 2020:4
39 CLSI. Determination of coagulation factor activities using the one-stage clotting assay. 2nd ed. CLSI guideline H48. Wayne, PA: Clinical and Laboratory Standards Institute;2016
40 Rosén S. Assay of factor VIII:C with a chromogenic substrate. Scand J Haematol Suppl 1984;40(Suppl 40):139–145
41 FIX R. 90 00 20 (package insert revision 04). SE-431 53 Sweden: Rossix AB Mölndal; 2014
42 FIX B. Package insert revision 11–2016. France: Hyphen Biomed Neuville sur Oise, 95000; 2016
43 Kershaw GW, Dissanayake K, Chen VM, Khoo TL. Evaluation of chromogenic factor IX assays by automated protocols. Haemophilia 2018;24(03):492–501
44 Suzuki A, Suzuki N, Kanematsu T, et al. Performance evaluation of Revohem™ FVIII chromogenic and Revohem™ FIX chromogenic in the CS-5100 autoanalyser. Int J Lab Hematol 2019;41(05):664–670
45 Hathaway WE, Christian MJ, Jacobson LJ. Variant mild haemophilia: discrepancy in one-stage and two-stage factor VIII assays. Thromb Haemost 1983;50:357
46 Duncan EM, Rodgers SE, McRae SJ. Diagnostic testing for mild hemophilia a in patients with discrepant one-stage, two-stage, and chromogenic factor VIII:C assays. Semin Thromb Hemost 2013;39(03):272–282
47 Schwaab R, Oldenburg J, Kemball-Cook G, et al. Assay discrepancy in mild haemophilia A due to a factor VIII missense mutation (Asn694Ile) in a large Danish family. Br J Haematol 2000;109(03):523–528
48 Bowyer AE, Van Veen JJ, Goodeve AC, Kitchen S, Makris M. Specific and global coagulation assays in the diagnosis of discrepant mild hemophilia A. Haematologica 2013;98(12):1980–1987
49 Poulsen AL, Pedersen LH, Hvas AM, Poulsen LH, Thykjaer H, Ingerslev J. Assay discrepancy in mild haemophilia A: entire population study in a national haemophilia centre. Haemophilia 2009;15(01):285–289
50 Pavlova A, Delev D, Pezeshkpoor B, Müller J, Oldenburg J. Haemophilia A mutations in patients with non-severe phenotype associated with a discrepancy between one-stage and chromogenic factor VIII activity assays. Thromb Haemost 2014;111(05):851–861
51 Pipe SW, Saenko EL, Eickhorst AN, Kemball-Cook G, Kaufman RJ. Hemophilia A mutations associated with 1-stage/2-stage activity discrepancy disrupt protein-protein interactions within the triplicated A domains of thrombin-activated factor VIIIa. Blood 2001;97(03):685–691

52 Pipe SW, Eickhorst AN, McKinley SH, Saenko EL, Kaufman RJ. Mild hemophilia A caused by increased rate of factor VIII A2 subunit dissociation: evidence for nonproteolytic inactivation of factor VIIIa in vivo. Blood 1999;93(01):176–183

53 Hakeos WH, Miao H, Sirachainan N, et al. Hemophilia A mutations within the factor VIII A2-A3 subunit interface destabilize factor VIIIa and cause one-stage/two-stage activity discrepancy. Thromb Haemost 2002;88(05):781–787

54 Michnick DA, Pittman DD, Wise RJ, Kaufman RJ. Identification of individual tyrosine sulfation sites within factor VIII required for optimal activity and efficient thrombin cleavage. J Biol Chem 1994;269(31):20095–20102

55 Mumford AD, Laffan M, O'Donnell J, et al. A Tyr346–>Cys substitution in the interdomain acidic region a1 of factor VIII in an individual with factor VIII:C assay discrepancy. Br J Haematol 2002;118(02):589–594

56 Bowyer AE, Goodeve A, Liesner R, Mumford AD, Kitchen S, Makris M. p.Tyr365Cys change in factor VIII: haemophilia A, but not as we know it. Br J Haematol 2011;154(05):618–625

57 Lyall H, Hill M, Westby J, Grimley C, Dolan G. Tyr346–>Cys mutation results in factor VIII:C assay discrepancy and a normal bleeding phenotype - is this mild haemophilia A? Haemophilia 2008;14(01):78–80

58 O'Brien DP, Tuddenham EGD. Purification and characterization of factor VIII 1,689-Cys: a nonfunctional cofactor occurring in a patient with severe hemophilia A. Blood 1989;73(08): 2117–2122

59 Gitschier J, Kogan S, Levinson B, Tuddenham EGD. Mutations of factor VIII cleavage sites in hemophilia A. Blood 1988;72(03): 1022–1028

60 Kihlberg K, Strandberg K, Rosén S, Ljung R, Astermark J. Discrepancies between the one-stage clotting assay and the chromogenic assay in haemophilia B. Haemophilia 2017;23(04): 620–627

61 Truedsson Å, Schmidt DE, Strålfors A, Soutari N, Norberg E, Letelier A. One-stage versus chromogenic factor IX activity in haemophilia B. Res Pract Thromb Haemost 2020;4(Suppl 1): [abstract]

62 Pouplard C, Trossaert M, LE Querrec A, Delahousse B, Giraudeau B, Gruel Y. Influence of source of phospholipids for APTT-based factor IX assays and potential consequences for the diagnosis of mild haemophilia B. Haemophilia 2009;15(01):365–368

63 Caron C, Dautzenberg MD, Delahousse B, et al. A blinded in vitro study with Refacto mock plasma samples: similar FVIII results between the chromogenic assay and a one-stage assay when using a higher cephalin dilution. Haemophilia 2002;8(05): 639–643

64 Jacquemin M, Vodolazkaia A, Toelen J, et al. Measurement of B-domain-deleted ReFacto AF activity with a product-specific standard is affected by choice of reagent and patient-specific factors. Haemophilia 2018;24(04):675–682

65 Mikaelsson M, Oswaldsson U. Assaying the circulating factor VIII activity in hemophilia A patients treated with recombinant factor VIII products. Semin Thromb Hemost 2002;28(03): 257–264

66 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-center study. J Thromb Haemost 2016;14(07):1428–1435

67 Wilmot HV, Hogwood J, Gray E. Recombinant factor IX: discrepancies between one-stage clotting and chromogenic assays. Haemophilia 2014;20(06):891–897

68 Gray E, Kitchen S, Bowyer A, et al. Laboratory measurement of factor replacement therapies in the treatment of congenital haemophilia: a United Kingdom Haemophilia Centre Doctors' Organisation guideline. Haemophilia 2020;26(01):6–16

69 Bolton-Maggs PH, Pasi KJ. Haemophilias A and B. Lancet 2003; 361(9371):1801–1809

70 Leong L, Evans V, Ramsey P, et al. Evaluation of methods for potency testing of pegylated FVIII (PEG-FVIII, Bay 94–9027). J Thromb Haemost 2011;9(Suppl. 2): Poster P-TU-223

71 Stennicke HR, Kjalke M, Karpf DM, et al. A novel B-domain O-glycoPEGylated FVIII (N8-GP) demonstrates full efficacy and prolonged effect in hemophilic mice models. Blood 2013;121 (11):2108–2116

72 Østergaard H, Bjelke JR, Hansen L, et al. Prolonged half-life and preserved enzymatic properties of factor IX selectively PEGylated on native N-glycans in the activation peptide. Blood 2011; 118(08):2333–2341

73 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(04):634–644

74 Santagostino E, Negrier C, Klamroth R, et al. Safety and pharmacokinetics of a novel recombinant fusion protein linking coagulation factor IX with albumin (rIX-FP) in hemophilia B patients. Blood 2012;120(12):2405–2411

75 Kitchen S, Jennings I, Makris M, Kitchen DP, Woods TAL, Walker I. Chromogenic and one stage FIX assays in the presence of Idelvion (rFIXFP), Alprolix (rFIXFc), Benefix and Replenine: data from a UK NEQAS for Blood Coagulation Survey (OC 65.2). Res Pract Thromb Haemost 2017;1:124–125

76 Peters RT, Low SC, Kamphaus GD, et al. Prolonged activity of factor IX as a monomeric Fc fusion protein. Blood 2010;115(10): 2057–2064

77 Shapiro AD, Ragni MV, Valentino LA, et al. Recombinant factor IX-Fc fusion protein (rFIXFc) demonstrates safety and prolonged activity in a phase 1/2a study in hemophilia B patients. Blood 2012;119(03):666–672

78 Nederlof A, Kitchen S, Meijer P, et al. Performance of factor IX extended half-life product measurements in external quality control assessment programs. J Thromb Haemost 2020;18(08): 1874–1883

79 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(01):138–148

80 Pickering W, Hansen M, Kjalke M, Ezban M. Factor VIII chromogenic assays can be used for potency labeling and postadministration monitoring of N8-GP. J Thromb Haemost 2016;14(08): 1579–1587

81 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 and chromogenic assay kits: a two-centre study. Haemophilia 2017;23(03): 458–465

82 Church N, Leong L, Katterle Y, et al. Factor VIII activity of BAY 94-9027 is accurately measured with most commonly used assays: results from an international laboratory study. Haemophilia 2018;24(05):823–832

83 Bowyer A, Key N, Dalton D, Kitchen S, Makris M. The coagulation laboratory monitoring of Afstyla single-chain FVIII concentrate. Haemophilia 2017;23(05):e469–e470

84 St Ledger K, Feussner A, Kalina U, et al. International comparative field study evaluating the assay performance of AFSTYLA in plasma samples at clinical hemostasis laboratories. J Thromb Haemost 2018;16(03):555–564

85 Afstyla Antihemophilic factor (recombinant), single chain US prescribing information. Rev April 2021. CSL Behring GmbH, Marburg, Germany. Accessed 16 November 2022 at: https://labeling.cslbehring.com/PI/US/Afstyla/EN/Afstyla-Prescribing-Information.pdf

86 Konkle BA, Shapiro AD, Quon DV, et al. BIVV001 fusion protein as factor VIII replacement therapy for hemophilia A. N Engl J Med 2020;383(11):1018–1027

87 Negrier C, Knobe K, Tiede A, Giangrande P, Møss J. Enhanced pharmacokinetic properties of a glycoPEGylated recombinant

factor IX: a first human dose trial in patients with hemophilia B. Blood 2011;118(10):2695–2701
88 Tiefenbacher S, Bohra R, Amiral J, et al. 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 (10):1901–1912
89 Ledger K, Feussner A, Kalina U, et al. Performance of a recombinant fusion protein linking coagulation factor IX with albumin (rIX-FP) in the one-stage assay. Haemophilia 2016;22:60
90 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(05):932–940
91 Bowyer AE, Shepherd MF, Kitchen S, Maclean RM, Makris M. Measurement of extended half-life recombinant factor IX products in clinical practice. Int J Lab Hematol 2019;41(02):e46–e49
92 Peyvandi F, Kenet G, Pekrul I, Pruthi RK, Ramge P, Spannagl M. Laboratory testing in hemophilia: impact of factor and non-factor replacement therapy on coagulation assays. J Thromb Haemost 2020;18(06):1242–1255
93 Jeanpierre E, Pouplard C, Lasne D, et al; French Study Group on the Biology of Hemorrhagic Diseases (the BIMHO group) Factor VIII and IX assays for post-infusion monitoring in hemophilia patients: guidelines from the French BIMHO group (GFHT). Eur J Haematol 2020;105(02):103–115
94 Kitazawa T, Igawa T, Sampei Z, et al. A bispecific antibody to factors IXa and X restores factor VIII hemostatic activity in a hemophilia A model. Nat Med 2012;18(10):1570–1574
95 Lauritzen B, Bjelke M, Björkdahl O, et al. A novel next-generation FVIIIa mimetic, Mim8, has a favorable safety profile and displays potent pharmacodynamic effects: results from safety studies in cynomolgus monkeys. J Thromb Haemost 2022;20(06): 1312–1324
96 EMA Accessed August 22, 2018 at: https://www.ema.europa.eu/en/documents/product-information/hemlibra-epar-product-information_en.pdf
97 Lenting PJ, Denis CV, Christophe OD. Emicizumab, a bispecific antibody recognizing coagulation factors IX and X: how does it actually compare to factor VIII? Blood 2017;130(23):2463–2468
98 Bowyer AE, Kitchen S, Maclean RM. The effect of emicizumab on assays of factor VIII activity in severe haemophilia A patients and artificially spiked plasma (PO27). Haemophilia 2019;25(Suppl 1):49
99 Adamkewicz JI, Chen DC, Paz-Priel I. Effects and interferences of emicizumab, a humanised bispecific antibody mimicking activated factor VIII cofactor function, on coagulation assays. Thromb Haemost 2019;119(07):1084–1093
100 Bowyer A, Ezban M, Kitchen S. Measuring the FVIII mimetic activity of the new bispecific antibody, Mim8, in severe haemophilia A plasma using APTT and one-stage FVIII assays. [abstract] Res Pract Thromb Haemost 2021;5(Suppl 2)
101 Adamkewicz J, Soeda T, Kotani N, Calatzis A, Levy G. Effect of emicizumab (ACE910) – a humanized bispecific antibody mimicking FVIII cofactor function – on coagulation assays commonly in use for monitoring of hemophilia A patients. Haemophilia 2017;23(Suppl 3):4
102 Jenkins PV, Bowyer A, Burgess C, et al. Laboratory coagulation tests and emicizumab treatment A United Kingdom Haemophilia Centre Doctors' Organisation guideline. Haemophilia 2020;26 (01):151–155
103 MASAC Recommendation on the Use and Management of Emicizumab-KXWH (HEMLIBRA®) for Hemophilia A with and without Inhibitors. National Hemophilia Foundation; 2020
104 Tripodi A, Chantarangkul V, Novembrino C, et al. Emicizumab, the factor VIII mimetic bi-specific monoclonal antibody and its measurement in plasma. Clin Chem Lab Med 2020;59(02): 365–371(CCLM)
105 Bowyer A, Kitchen S, Maclean R. Measurement of antifactor VIII antibody titre in the presence of emicizumab; use of chromogenic Bethesda assays. Int J Lab Hematol 2021;43(04): O204–O206
106 Machin N, Ragni MV. An investigational RNAi therapeutic targeting antithrombin for the treatment of hemophilia A and B. J Blood Med 2018;9:135–140
107 Shapiro AD, Angchaisuksiri P, Astermark J, et al. Subcutaneous concizumab prophylaxis in hemophilia A and hemophilia A/B with inhibitors: phase 2 trial results. Blood 2019;134(22): 1973–1982
108 Patel-Hett S, Martin EJ, Mohammed BM, et al. Marstacimab, a tissue factor pathway inhibitor neutralizing antibody, improves coagulation parameters of ex vivo dosed haemophilic blood and plasmas. Haemophilia 2019;25(05):797–806
109 Polderdijk SGI, Baglin TP, Huntington JA. Targeting activated protein C to treat hemophilia. Curr Opin Hematol 2017;24 (05):446–452
110 Peyvandi F, Garagiola I. Clinical advances in gene therapy updates on clinical trials of gene therapy in haemophilia. Haemophilia 2019;25(05):738–746
111 National Institute of Health U.S. National Library of Medicine. Accessed August 22, 2020 at: https://clinicaltrials.gov
112 Pipe SW, Gonen-Yaacovi G, Segurado OG. Hemophilia A gene therapy: current and next-generation approaches. Expert Opin Biol Ther 2022;22(09):1099–1115
113 U.S. Department of Health and Human Services, FDA, Center for Biologics Evaluation and Research Human Gene Therapy for Hemophilia: Guidance for Industry. Accessed August 22, 2020 at: https://www.fda.gov/regulatory-information/search-fda-guidance-documents/human-gene-therapy-hemophilia
114 Rangarajan S, Walsh L, Lester W, et al. AAV5-factor VIII gene transfer in severe hemophilia A. N Engl J Med 2017;377(26): 2519–2530
115 Konkle BA, Stine K, Visweshwar N, et al. Updated follow-up of the Alta Study, a phase 1/2, open label, adaptive, dose-ranging study to assess the safety and tolerability of SB-525 gene therapy in adult patients with severe hemophilia A. [abstract]. Blood 2019; 134(Supplement 1): 2060
116 Pasi KJ, Rangarajan S, Mitchell N, et al. Multiyear follow-up of AAV5-hFVIII-SQ gene therapy for hemophilia A. N Engl J Med 2020;382(01):29–40
117 Lengler J, Gritsch H, Weiller M, Scheiflinger F, Hoellriegl W, Rottensteiner H. Factor VIII clotting and chromogenic activities for TAK- 754 / SHP654, a clinical hemophilia A gene therapy candidate, using in vitro and in vivo assays. [abstract]Res Pract Thromb Haemost 2019;3:303
118 Mikaelsson M, Oswaldsson U, Sandberg H. Influence of phospholipids on the assessment of factor VIII activity. Haemophilia 1998;4(04):646–650
119 Rosen S, Tiefenbacher S, Robinson M, et al. Activity of transgene-produced B-domain-deleted factor VIII in human plasma following AAV5 gene therapy. Blood 2020;136(22):2524–2534
120 Simioni P, Tormene D, Tognin G, et al. X-linked thrombophilia with a mutant factor IX (factor IX Padua). N Engl J Med 2009;361 (17):1671–1675
121 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(05):1212–1218
122 Foley JH, Kitchen S, Shehu E, et al. Multi-centre field study of one-stage and chromogenic factor IX assays in samples containing the factor IX Padua variant. [abstract]Res Pract Thromb Haemost 2020;4(01):x
123 Shehu E, Goodale A, Allen O, et al. Mechanistic evaluation of factor IX-Padua activity in chromogenic FIX and thrombin generation assays. [abstract]Res Pract Thromb Haemost 2020; 4(01):x

124 Robinson M, George LA, Samuelson-Jones BJ, et al. Activity of a FIX-Padua transgene product in commonly used FIX:C one-stage and chromogenic assay systems following PF-06838435 (SPK-9001) gene delivery. Blood 2018;132(Suppl 1):2198
125 Lee C, Barrowcliffe T, Bray G, et al. Pharmacokinetic in vivo comparison using 1-stage and chromogenic substrate assays with two formulations of Hemofil-M. Thromb Haemost 1996;76(06):950–956
126 Barrowcliffe TW, Raut S, Hubbard AR. Discrepancies in potency assessment of recombinant FVIII concentrates. Haemophilia 1998;4(04):634–640
127 Sommer JM, Moore N, McGuffie-Valentine B, et al. Comparative field study evaluating the activity of recombinant factor VIII Fc fusion protein in plasma samples at clinical haemostasis laboratories. Haemophilia 2014;20(02):294–300
128 Bowyer AE, Ezban M, Kitchen S. Measuring the chromogenic FVIII mimetic activity of the new bispecific antibody, Mim8, in artificially spiked severe haemophilia A plasma. [abstract]Res Pract Thromb Haemost 2021:PB0679
129 Trossaërt M, Regnault V, Sigaud M, Boisseau P, Fressinaud E, Lecompte T. Mild hemophilia A with factor VIII assay discrepancy: using thrombin generation assay to assess the bleeding phenotype. J Thromb Haemost 2008;6(03):486–493
130 Bowyer AE, Duncan EM, Antovic JP. Role of chromogenic assays in haemophilia A and B diagnosis. Haemophilia 2018;24(04):578–583
131 EMA Accessed August 23, 2022 at: https://www.ema.europa.eu/en/documents/product-information/elocta-epar-product-information_en.pdf
132 EMA Accessed August 23, 2022 at: https://www.ema.europa.eu/en/documents/product-information/afstyla-epar-product-information_en.pdf
133 EMA Accessed August 23, 2022 at: https://www.ema.europa.eu/en/documents/product-information/adynovi-epar-product-information_en.pdf
134 EMA Accessed August 23, 2022 at: https://www.ema.europa.eu/en/documents/product-information/jivi-epar-product-information_en.pdf
135 EMA Accessed August 23, 2022 at: https://www.ema.europa.eu/en/documents/product-information/esperoct-epar-product-information_en.pdf
136 EMA Accessed August 23, 2022 at: https://www.ema.europa.eu/en/documents/product-information/idelvion-epar-product-information_en.pdf
137 EMA Accessed August 23, 2022 at: www.ema.europa.eu/en/documents/product-information/alprolix-epar-product-information_en.pdf
138 EMA Accessed August 23, 2022 at: https://www.ema.europa.eu/en/documents/product-information/refixia-epar-product-information_en.pdf