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REVIEW ARTICLE

Laboratory testing in hemophilia: Impact of factor and non-factor replacement therapy on coagulation assays

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Abstract
The advent of extended half-life (EHL) recombinant clotting factors and innovative non-factor replacement therapeutics, such as emicizumab, offers several advantages over existing products for the prophylactic treatment of people living with hemophilia (PwH). These include low annual bleeding rates with less frequent dosing, higher trough plasma concentrations, and a more convenient route of administration. However, increasing use of these therapies poses challenges to clinicians and coagulation laboratories due to the lack of standardized assays for monitoring of hemostatic parameters, and the potential for misinterpretation of test results, which may jeopardize patient safety. Definitive diagnosis of hemophilia and treatment monitoring is reliant on demonstrating factor VIII (FVIII; hemophilia A) or factor IX (FIX; hemophilia B) deficiency using a functional coagulation assay. The most frequently used assays are based on activated partial thromboplastin time, using a one-stage or two-stage process. While one-stage and chromogenic assays have performed well with human-derived FVIII and FIX and full-length recombinant products, EHL recombinant factors are heterogeneous in structure and mode of action and therefore show wide variation in activity levels between different one-stage assays, and between one-stage and chromogenic assays. In the context of the recommended stepwise approach for laboratory diagnosis of hemophilia, we examine the diagnostic challenges associated with the use of EHL factors and novel non-factor therapeutics and consider the optimal diagnostic approach in PwH who are receiving these treatments. Ultimately, accurate diagnostic solutions are a prerequisite for personalized therapy to minimize treatment burden and improve quality of life in PwH.

KEYWORDS
blood coagulation factors, emicizumab, factor VIII, factor IX, hemophilia A, hemophilia B

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1 | BACKGROUND

Hemophilia results from a deficiency of coagulation factor VIII (FVIII; hemophilia A) or factor IX (FIX; hemophilia B). Accurate diagnosis of hemophilia is essential to inform appropriate management of people living with hemophilia (PwH) and can only be made with the support of a comprehensive laboratory service.[1-4] As highlighted in World Federation of Hemophilia guidelines, correct diagnosis is dependent on laboratories following strict protocols and procedures, which requires knowledge and expertise in coagulation laboratory testing, use of the correct equipment and reagents, and quality assurance.[1]

Effective management of hemophilia involves preventing and treating bleeding episodes by administering the deficient clotting factor or using new treatment approaches such as emicizumab (for hemophilia A), preferably in a comprehensive care setting.[1] The current cornerstone of management is factor replacement therapy,[1,3] which is associated with potential infusion-related side effects, including pyrexia, headache, and immunologic reactions. Of particular importance is the development of neutralizing anti-FVIII antibodies (inhibitors) that significantly reduce the effectiveness of FVIII treatment and complicate the therapeutic management of patients. Thus, accurate and precise clotting factor assays and inhibitor testing are essential for optimal management of hemophilia. Guidelines also recommend that laboratory specialists should work alongside the medical director, nurse coordinator, musculoskeletal expert, and psychosocial expert to form a multidisciplinary core team responsible for delivering comprehensive care for PwH.[1]

Accurate tailoring of therapy for PwH also requires regular laboratory follow-up to determine specific trough and peak levels of replacement factors for each individual.[5,6] Early studies found that joint disease in PwH can be minimized through a prophylactic treatment approach by preventing FVIII or FIX concentrations from falling below 1% (ie, the detection limit),[7] indicating that the incidence of joint bleeds is associated with trough levels of administered therapeutics. The probability of spontaneous joint bleeds decreases with an increase of FVIII activity.[5,6] Given that as few as three consecutive joint bleeds can expose joints to irreversible harm,[8] modern factor replacement therapy aims to achieve a zero-bleeds strategy. More recent work has highlighted significant individual variation in the pharmacokinetics of replacement factor concentrates, providing further rationale for pharmacokinetic-based personalized prophylactic therapy.[9,10] Modern regimes take bleeding pattern, condition of the musculoskeletal system, level and timing of physical activity, and blood coagulation factor levels into account;[11] considering these aspects some patients may need to achieve higher trough levels (ie, exceeding 1%-3%).

In pursuit of the goal of tailored therapy, electronic apps are increasingly being used by patients to track bleeds and factor levels, and a newer generation of extended half-life (EHL) factor products have been developed (Table 1), offering several advantages over standard products, including a lower frequency of dosing and higher trough plasma concentrations.[3,12] However, the availability of EHL factors raises new issues of accuracy and reproducibility for laboratory testing, centered around the ability of laboratories to achieve comparable activity measurements for post-infusion samples.[13] A number of non-factor replacement molecules have also been developed, such as emicizumab, which is approved for prophylaxis of hemorrhage in patients with hemophilia A with or without FVIII inhibitors in numerous countries worldwide, including Europe, the United States, Japan, and Australia.[14,15] Laboratory monitoring of coagulation status during emicizumab therapy can help to rule out anti-drug antibodies (ADAs) in the event of breakthrough spontaneous bleeding, as part of a pre-operative assessment to determine adequacy of hemostasis for surgical procedures, or in cases in which co-administration of factor concentrates is necessary. However, as the mechanism of action of emicizumab differs from FVIII this has implications for the selection of diagnostic assay and interpretation of laboratory results, whereby product-specific calibration is required to enable therapeutic drug monitoring. Additionally, laboratory monitoring with differential application of specific FVIII single-factor assays can discriminate endogenous FVIII activity from that associated with FVIII replacement products and emicizumab.

Other novel therapeutic approaches may also present challenges for clinical laboratories. Non-factor replacement molecules fitusiran and concizumab do not target the intrinsic tenase complex and are being developed specifically for the treatment of both hemophilia A and B; these agents are currently being investigated in phase II/III trials,[16-19] in which their net effect on the restoration of hemostasis is demonstrated by an indirect or direct dose-dependent increase in thrombin generation.[16-17,20] Advances in gene therapy also offer hope to PwH,[21,22] but may necessitate long-term monitoring of factor levels to confirm continued efficacy. Ultimately, while the development of innovative coagulant agents has the potential to reduce treatment burden and increase the quality of life for PwH, methods for precise monitoring of hemostatic parameters with these products/approaches need to evolve in parallel.[20,23,24]

In summary, the realization of personalized treatment regimens with EHL factor preparations and novel therapeutics such as emicizumab requires the availability of precision diagnostic approaches for accurate measurement of these agents. In this review, we aim to provide education around the optimal diagnostic and therapeutic monitoring approach in PwH who are receiving treatment with EHL factors or emicizumab.

2 | DIAGNOSTIC PATHWAYS IN HEMOPHILIA

2.1 | Screening tests

Current guidelines recommend a stepwise approach in patients presenting with symptoms consistent with spontaneous bleeding (Figure 1). Initial screening tests to identify the cause of bleeding include the activated clotting time (aCT) and activated partial thromboplastin time (aPTT) tests.[1]

aCT is a point-of-care test whereby blood samples are collected per standard guidelines,[1,25] and immediately added to a surface

TABLE 1 Novel therapeutics for hemophilia including emicizumab and extended half-life products *[Adapted from Lambert et al, Practical aspects of extended half-life products for the treatment of haemophilia. Ther Adv Hematol. 2018; 9:295-308; Copyright © 2018, with permission from © SAGE Publications]*12

Disease	Molecule name	Brand/generic name	Structure	Mean terminal half-life	Company	Status
Hemophilia A	rFVIII-Fc	Eloctate®/Elocta	rBDD-FVIII Fc fusion	19 hours	Biogen, Inc. and Sobi	Approved in USA, Canada, Europe, Australia, New Zealand, and Japan
	BAX-855	Adynovate®/Adynovi	PEGylated rFVIII (20kDa)	14-16 hours	Shire	Approved in USA, Japan, Canada, Switzerland, Colombia, and Europe
	rFVIII-SC	Afstyla®	rFVIII-SC	14 hours	Behring	Approved in USA, Canada, Europe, Australia, New Zealand, and Japan
	BAY94-9027	Damoctocog alfa pegol	PEGylated rBDD-rFVIII (60kDa)	19 hours	Bayer HealthCare Pharmaceuticals	Under development
	N8-GP	Esperoct®	GlycoPEGylated rBDT-FVIII (40kDa)	18-19 hours	Novo Nordisk	Approved in Europe, USA, and Canada
	Emicizumab	Hemlibra®	Bispecific mAb	26.7 days[a]	Roche	Approved in Europe, USA, Japan, Australia, and other countries
Hemophilia B	rFIX-Fc	Alprolix®	rFIX-Fc fusion	82 hours	Biogen, Inc. and Sobi	Approved in USA, Canada, Europe, Japan, Australia, New Zealand, and other countries
	rFIX-FP (CSL654)	Idelvion®	rFIX-albumin fusion	102 hours	CSL Behring	Approved in USA, Europe, Canada, and Japan
	N9-GP	Refixia®/Rebinyn®	GlycoPEGylated rFIX	93 hours	Novo Nordisk	Approved in Europe and USA (on-demand treatment)

Abbreviations: Fc, fragment crystallizable; FIX, factor IX; FVIII, factor VIII; mAb, monoclonal antibody; PEG, polyethylene glycol; rBDD, recombinant B-domain deleted; rBDT, recombinant B-domain truncated; rFIX, recombinant factor IX; rFVIII, recombinant factor VIII; SC, single chain; Sobi, Swedish Orphan Biovitrum AB (publ) (Sobi™).

[a]Following intravenous administration of 0.25 mg/kg in healthy subjects.

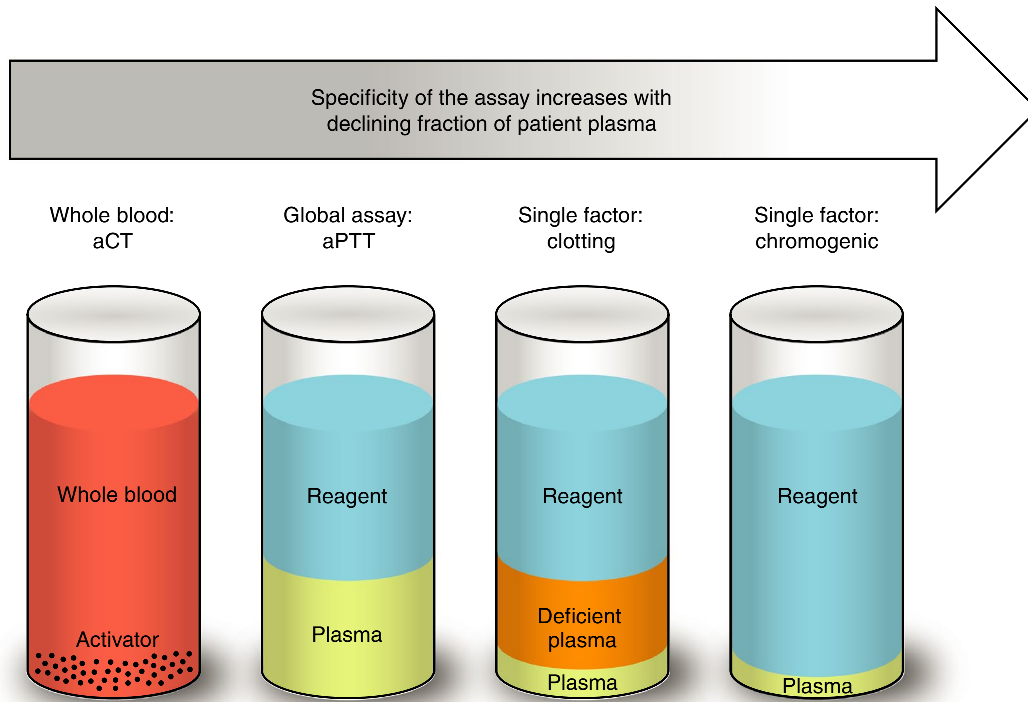

FIGURE 1 Stepwise approach for laboratory testing of blood and plasma samples in patients presenting with bleeding symptoms. aCT, activated clotting time; aPTT, activated partial thromboplastin time; POCT, point-of-care test

activator (eg, kaolin); the time to clot formation is known as the aCT and is sensitive to deficiencies in factors VIII (<25% of normal), IX, X, XI, and XII. aCT is mostly used for anticoagulation monitoring, being sensitive to parenteral and oral anticoagulants (Xa/IIa antagonists); a limitation is that the test is sensitive to platelet count and hemodilution.26 Due to low sensitivity and specificity, aCT is not recommended for diagnosis and monitoring of hemophilia patients under elective conditions.

aPTT is a standard laboratory test and is more precise than aCT. Evaluation of aPTT involves incubation of platelet poor plasma (PPP) with a phospholipid reagent and subsequent addition of a surface activator; the same process is performed in parallel using control plasma.[27] A prolonged aPTT in the presence of a normal prothrombin time and platelet count is suggestive of hemophilia.[1] However, several variables can affect the measurement of aPTT (Figure 2), which is also prolonged by any deficiency of the intrinsic (contact) pathway (eg, deficiencies of factors XII, XI, and IX) and common coagulation pathway (eg, deficiencies of factors V, X, II, or fibrinogen) and in the presence of inhibitors. Measurement techniques may also influence aPTT; for example, reagents with different sensitivities are available, and deviation from manufacturer recommended incubation times or the use of different coagulometers can affect clotting times.[27,28]

Subsequent correction or inhibition on mixing studies using pooled normal plasma can help to determine whether the prolonged aPTT is due to coagulation factor deficiency or inhibitors (eg, circulating lupus anticoagulants, or specific coagulation factor inhibitors), respectively. Mixing studies with FVIII- or FIX-deficient plasma can be used to distinguish between hemophilia A/B if a factor assay is not available.[1]

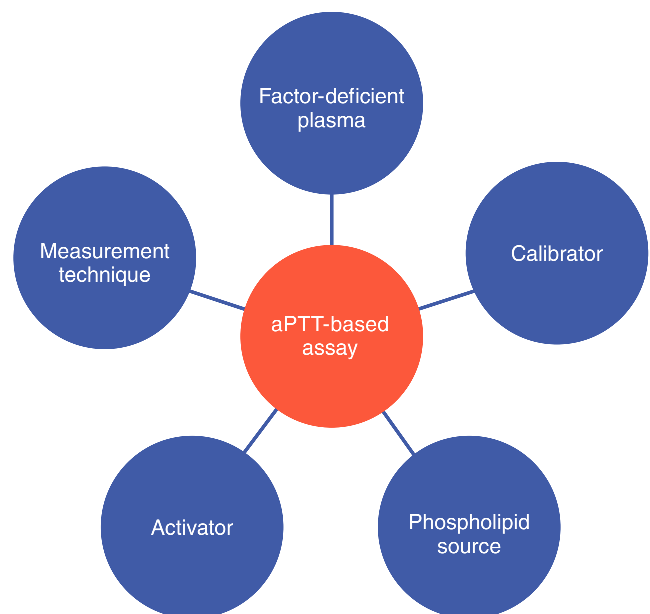

FIGURE 2 Variables affecting measurement of aPTT. aPTT, activated partial thromboplastin time

2.2 | Confirmation tests

Definitive diagnosis of hemophilia and monitoring of treatment depends on demonstration of FVIII or FIX deficiency by functional coagulation assay (FVIII:C or FIX:C) and exclusion of differential diagnoses such as von Willebrand disease.[1-4] Coagulation assays

can be used to determine the severity of hemophilia, to check coagulation status prior to surgery, and for dose optimization during prophylactic treatment in which a decrease in factor activity during adequate treatment may indicate inhibitor development.

One-stage assays based on aPTT are the most commonly used functional coagulation assays and are widely available in clinical laboratories.[1,4,27] The assay compares the ability of platelet-poor citrated test plasma (ie, patient sample) and standard plasma to correct the aPTT of a plasma that is totally deficient in FVIII or FIX but contains all other components of the normal clotting process.[27] Samples are pre-diluted with assay buffer before addition of aPTT reagent, comprising phospholipid plus contact activator, and sample FVIII or FIX activity determines coagulation time. Samples may also be pre-diluted with FVIII- or FIX-deficient plasma to construct low curves when very low levels are measured. Assay performance is affected by the type of contact activator (that varies with each aPTT reagent), factor-deficient plasma, and the instrument on which the assay is performed. Different aPTT reagents have varying sensitivities to coagulation factor deficiencies, detection of lupus anticoagulants, and heparin sensitivities. The large majority of aPTT reagents will be abnormal in moderate and severe hemophilia; however, not all reagents will detect mild hemophilia. Thus, for diagnosing hemophilia it is important to select an aPTT reagent with proven capacity to detect all hemophilia categories (ie, mild to severe hemophilia).4 In addition, it is important for each performing laboratory to know the sensitivity of their reagent to detection of FVIII and FIX deficiency on their respective instruments.

A prolonged aPTT that is not fully corrected into the laboratory-established reference range by mixing patient plasma with an equal volume of normal plasma suggests the presence of an inhibitor, such as lupus anticoagulant or neutralizing anti-FVIII or anti-FIX antibodies. Confirmation that an inhibitor is directed against a clotting factor requires a specific inhibitor assay.[1] The Bethesda assay compares activity in a mix of normal plasma and patient plasma with heat inactivation of FVIII or FIX activity versus a mix of control and normal plasma to determine the percentage of FVIII/FIX activity inhibited by a patient's plasma.[29] The Nijmegen modification of the Bethesda assay increases sensitivity.[30,31] One Bethesda unit (BU) is defined as the amount of an inhibitor that will neutralize half of 1 unit of FVIII:C in normal plasma after a 2-hour incubation at 37°C, and an inhibitor titer of ≥0.6 BU/mL (or ≥0.5 BU/mL following heat treatment) should be considered clinically relevant.[1] The Bethesda assay might be performed in several clinical scenarios, including in previously untreated patients to confirm reduced recovery after factor administration, for prognostic considerations (ie, determination of high or low responders) and therapeutic monitoring of immune tolerance induction, and for surveillance when switching factors or switching from factor- to non-factor-based regimens (particularly after intermittent application of FVIII).

Limitations of one-stage assays include susceptibility to interference from pre-analytical sample handling and processing (eg, pre-activation of FVIII), anti-phospholipid antibodies, or heparin contamination, and the potential for misleading results.[32,33] For example, in individuals with mild hemophilia A and genetic mutations of FVIII, FVIII:C measured by a one-stage assay may be higher than when measured using a two-stage aPTT-based assay (which is rarely used or available in clinical laboratories) or chromogenic substrate assay (CSA; which is also based on the two-stage procedure) and may appear normal; the reverse is also possible.[33]

The CSA is a two-stage assay based on the principle that the amount of FVIII is rate-limiting during clotting of a test mixture containing FX in excess, activated FIX (FIXa), phospholipid, and calcium ions.[27,34] PPP is incubated with the reagent cocktail at 37°C and thrombin. Color formation is directly proportional to FVIII:C or FIX:C activity in the PPP sample and can be measured spectrophotometrically.[27] FX in the reagent can be of human or bovine origin, which has implications for the assessment of coagulation status during emicizumab therapy (as discussed in more detail below). CSAs are generally more sensitive to mildly lowered factor activity and are more specific than one-stage assays due to the high dilution factor of the sample,[4,32] although for some FVIII mutations chromogenic and one-stage assays can complement each other.[35] While chromogenic assay kits are commercially available, they are not uniformly approved by regulatory agencies such as the US Food and Drug Administration (FDA).

A chromogenic version of the Bethesda assay has proven successful in testing for the presence of inhibitors,[36] and can determine FVIII inhibitor titer in the presence of emicizumab, as discussed below.[37]

3 | EFFECTS OF EXTENDED HALF-LIFE FACTORS OR EMICIZUMAB ON DIAGNOSTIC PERFORMANCE OF FACTOR ASSAYS

3.1 | Influence of extended half-life factors on commonly used laboratory tests for hemophilia

Three key molecular strategies have been used to extend the half-lives of recombinant FVIII (rFVIII) and recombinant FIX (rFIX) preparations: attachment of polyethylene glycol (PEG; PEGylation); fusion to other proteins such as recombinant human albumin or the fragment crystallizable (Fc) region of human immunoglobulin G1 (IgG1); and development of a single-chain protein (Table 1).[12] PEG conjugation increases the circulation time of FVIII and FIX by protecting against enzymatic degradation; similarly, fusion to the Fc-part of IgG or to albumin protects FVIII and FIX from lysosomal sorting and degradation.[39] While most commercially available FVIII concentrates consist of two chains, the rFVIII single-chain (rFVIII-SC) preparation comprises a truncated B-domain that covalently links the heavy and light chains to improve the pharmacokinetic properties of the molecule.[39] The half-lives of EHL-rFIX products can be extended four- to six-fold using these technologies, while the half-life extension of EHL-rFVIII products is restricted to 1.5- to two-fold due to their interaction with von Willebrand factor.[39]

A number of PEGylated FVIII and FIX products have been investigated in hemophilia clinical trials, or are currently in development. Rurioctocog alfa pegol (BAX-855; Adynovate®), a PEGylated form of full-length rFVIII, is licensed for the prevention and treatment of hemorrhage in hemophilia A[40]; nonacog beta pegol (N9-GP; Refixia®), a PEGylated version of the FIX activation peptide, is approved for the prevention and treatment of hemorrhage in adult hemophilia B[41]; and turoctocog alfa pegol (N8-GP; Esperoct®), which contains a truncated B-domain attached to a PEG molecule, is approved for treatment and prophylaxis of bleeding in patients 12 years and above with hemophilia A.[42] Damoctocog alfa pegol (BAY94-9027), a PEGylated form of B-domain-depleted rFVIII molecule, is undergoing clinical assessment.13,39 In terms of fusion proteins, eftrenonacog alfa (rFIX-Fc; Alprolix®), a single recombinant FIX molecule fused to the Fc domain of IgG1, was the first EHL factor concentrate to gain FDA approval for the prophylaxis and treatment of hemophilia B[43]; albutrepenonacog alfa (rFIX-FP; Idelvion®), a fusion protein between rFIX and human albumin, was subsequently approved in this indication.[39,44] Lonoctocog alfa (rFVIII-SC; Afstyla®) is approved for the treatment of hemophilia A across a number of countries.[45]

Although one-stage and chromogenic assays have both performed well with human-derived FVIII and FIX and full-length recombinant products, EHL recombinant factors show wide variation in activity levels between different one-stage assays, and between one-stage and chromogenic assays.[24,46] In one-stage assays, these differences relate to the type of contact activator used, which is either silica, ellagic acid, kaolin, or polyphenolic acid.38,47 Therefore, for some EHL-rFVIII and EHL-rFIX products, certain one-stage assays are unsuitable for monitoring purposes.[38,47]

The International Prophylaxis Study Group (IPSG) recently produced a summary of one-stage assay results according to EHL concentrate and aPTT reagent, based on laboratory and clinical studies (Table 2). Of note, not all of the study designs were the same; some were field studies and others were limited to a single, or only a few, reference laboratories.[38,47] Looking at rFVIII concentrates, only rFVIII-Fc and rFVIII-PEG appeared to have no serious issues with any tested reagent, although this conclusion was based on findings from field studies only.[48] BAY94-9027 and N8-GP showed discrepant findings with respect to silica-based aPTT reagents: correct measurements were achieved with SynthASil® and Pathromtin® SL, but decreased recovery was observed with STA®-PTT Automate and PTT-SP and TriniCLOT™. The same was true for ellagic acid reagents, with correct measurements achieved with Actin® FS, Actin® FSL, and DG Synth, but decreased recovery observed with SynthaFax® (N8-GP only).[49-53] With regard to the FVIII-SC molecule, all aPTT reagents led to decreased recoveries.[54,55] Contrary to FDA guidance, IPSG and other groups suggest that when assessing FVIII-SC effects, it may not be appropriate to multiply the one-stage assay result by a factor of two; if chromogenic FVIII assays are available they should be preferably used.[13,49,56]

The measurement of rFIX concentrates with one-stage assays has been reported by the IPSG. Both rFIX-Fc and rFIX-FP were correctly measured using the selection of silica reagents tested, but both products resulted in decreased recoveries with kaolin reagents.[57,58] By contrast, N9-GP resulted in approximately four-fold overestimation of FIX levels when certain silica reagents were used,[59,60] and other reagents underestimated the effects of N9-GP (Actin FS, SyntaSIL, and STA®-CK Prest®).[61] N9-GP can be correctly measured using a polyphenolic acid reagent (CephaScreen) and one ellagic acid reagent (SynthaFax)62 (Table 2). Use of product-specific reference standards can reduce overestimation of BAX-855 recovery.[63]

Chromogenic FVIII assays can be used to measure the recoveries of all EHL-rFVIII concentrates[38,48,53,63-65] (Table 3), as they are associated with less variation than one-stage assays and measurements are more precise. However, data relating to the accuracy of chromogenic assays for measuring EHL FVIII agents are relatively limited and are often based on field studies. Furthermore, the accurate recovery of target analytes by chromogenic FVIII assays may be dependent on the use of product-specific references,[63] and an overestimation of N8-GP recovery has been reported with some assays.[49,53,64] Data for the two available rFIX chromogenic assays (Biophen™ Chromogen factor IX; Rox FIX) are relatively limited: N9-GP can be correctly measured using either of these assays[59]; rFIX-Fc can be correctly measured using the BioPhen assay57; rFIX-FP can be correctly measured using the Rox assay[66] (Table 3).

In addition to interference of coagulation activity tests, the presence of EHL-FVIII and EHL-FIX agents can make detection of low-titer FVIII and FIX inhibitors difficult using the Nijmegen Bethesda assay.[34] It is well established that heat activation and centrifugation can be used to eliminate interference from endogenous and therapeutic FVIII, and facilitate low-level inhibitor detection;[34] however, until recently the effectiveness of this pre-analytical protocol in samples containing EHL-FVIII and EHL-FIX agents had not been investigated. Two small studies have now demonstrated that molecular modification of FVIII and FIX does not impact heat inactivation.[67,68] The first study demonstrated that a 30-minute incubation at 56 resulted in heat inactivation of EHL-FVIII and allowed accurate determination of FVIII inhibitors using either one-stage or chromogenic assays.67 This finding was confirmed in the subsequent study, which also showed that pre-analytic heat treatment can remove interference by EHL-FIX agents.68 Collectively, these findings mean that patients do not need to undergo a washout period prior to inhibitor measurement.

3.2 | Guidelines for laboratories for monitoring the use of extended half-life factors

Guidance on the use of EHL coagulation factor concentrates in routine clinical practice was published in 2016 by the United Kingdom Haemophilia Centre Doctors' Organisation (UKHCDO).[13] Recommendations to laboratories for monitoring EHL factor concentrates included using an assay that has been validated for use with the specific EHL factor. This may be a chromogenic assay, a one-stage assay with a method shown to give appropriate results, or a one-stage assay with an appropriate high-quality product-specific

TABLE 2 Factor VIII and IX assay results (qualitative) in one-stage clotting assay according to EHL concentrate and aPTT reagent used *[Adapted from Young et al, Laboratory assay measurement of modified clotting factor concentrates: A review of the literature and recommendations for practice. J Thromb Haemost. 2019. 10.1111/jth.14394; Copyright © 2019, with permission from International Society on Thrombosis and Haemostasis]*[38]

	Factor VIII EHL concentrate					Factor IX EHL concentrate		
Reagent	**rFVIII Fc**	**BAX855 (rFVIII-PEG)**	**rFVIII-SC**	**N8-GP**	**BAY-94-9027**	**rFIX Fc**	**rFIX FP**	**N9-GP**
Silica reagents								
SynthaSIL	Correct	Correct	Underestimate	Correct	Correct	Correct	Correct	Underestimate
STA-PTT Automate	Correct	Correct	Underestimate	Underestimate	Underestimate	Unknown	Correct	Overestimate
PTT-SP	Unknown	Unknown	Underestimate	Underestimate	Underestimate	Unknown	Unknown	Overestimate
Pathromtin SL	Correct	Correct	Underestimate	Correct	Correct	Correct	Correct	Overestimate
TriniCLOT Auto	Correct	Correct	Underestimate	Underestimate[a]	Unknown	Correct	Unknown	Overestimate
TriniCLOT HS	Correct	Correct	Underestimate	Underestimate[a]	Unknown	Correct	Correct	Overestimate
Ellagic acid reagents								
Actin FS	Correct	Correct	Underestimate	Correct	Correct	Correct/high[b]	Underestimate	Underestimate
Actin FSL	Correct	Correct	Underestimate	Correct	Correct	Correct	Unknown	Underestimate
Synthafax	Unknown	Correct	Underestimate	Decreased	Correct	Correct	Unknown	Correct
DG Synth	Unknown	Unknown	Unknown	Correct	Unknown	Correct	Unknown	Correct
Kaolin reagents								
CK Prest	Correct	Correct	Underestimate	Correct	Unknown	Underestimate	Underestimate	Underestimate
Polyphenolic acid reagents								
Cephascreen	Correct	Correct	Underestimate	Correct	Correct	Correct	Unknown	Correct

Abbreviations: aPTT, activated partial thromboplastin time; EHL, extended half-life; Fc, fragment crystallizable; FIX, factor IX; PEG, polyethylene glycol; rFIX, recombinant factor IX; rFVIII, recombinant factor VIII; SC, single chain.

[a]Hansen and colleagues report that "TriniCLOT" underestimated N8-GP recovery by >30%[51]; TriniCLOT HS or TriniCLOT Auto was not specified.

[b]Correct in normal levels. But too high at FIX levels of 5%-20%.

TABLE 3 Factor VIII and IX assay results (qualitative) with chromogenic assays

	Factor VIII EHL concentrate					Factor IX EHL concentrate		
Assay	**rFVIII Fc**	**BAX855 (rFVIII-PEG)**	**rFVIII-SC**	**N8-GP**	**BAY-94-9027**	**rFIX Fc**	**rFIX FP**	**N9-GP**
Chromogenic FVIII assays								
Chromogenix Coatest SP4	Correct[a,48]	Unknown	Unknown	Correct/ overestimated[b,e,49,53,64]	–	–	–	–
Chromogenix Coamatic FVIII	Correct[a,48]	Correct[65]	Correct[c,53]	Correct/ overestimated[b,e,49,53]	–	–	–	–
Siemens FVIII chromogenic	Correct[a,48]	Correct[d,63,65]	Correct[c,53]	Correct [b,e49,53,64,]	–	–	–	–
Hyphen Biomed Biophen FVIII	Correct[a,48]	Correct[d,63]	Correct[c,53]	Overestimated[b,e,49,53,64]	–	–	–	–
Werfen HemosIL Electrachrome	Unknown	Correct[65]	Unknown	Correct/ overestimated[e,53]	–	–	–	–
Technoclone Technochrom FVIII:C	Correct[a,48]	Unknown	Unknown	Correct/ overestimated[e,53]	–	–	–	–
Berichrom FVIII	Unknown	Correct[65]	Unknown	Unknown	–	–	–	–
Chromogenic FIX assays								
Rossix AB ROX FIX	–	–	–	–	–	Unknown	Correct[66]	Correct
Hyphen BioPhen FIX	–	–	–	–	–	Correct[57]	Unknown	Correct[59]

Abbreviations: EHL, extended half-life concentrate; Fc, fragment crystallizable; FIX, factor IX; FVIII, factor VIII; IS, International Standard; PEG, polyethylene glycol; rFIX, recombinant factor IX; rFVIII, recombinant factor VIII; SC, single chain; WHO, World Health Organization.

[a]Mean recovery across assays was 119%, 133%, and 120% for 0.87, 0.22, and 0.054 IU/mL samples.[48]

[b]WHO 8th IS for FVIII concentrate calibrator produced results closer to expected values and approximately 10% lower than those obtained using a plasma calibrator.[64]

[c]Based on spiked samples at concentrations 0.30, 0.60, 1.00 IU/mL.[54]

[d]Recoveries ranged from 72.7% to 103.7% with use of a product-specific reference.63

[e]N8-GP recovery was consistent across all concentrations of spiked samples: 0.03 IU/mL (119.1%), 0.2 IU/mL (130.5%), 0.6 IU/mL (135.0%), 0.9 IU/mL (132.7%); overall mean recovery was within the upper limit of the acceptable range (129%, 95% CI 123-136).[53]

TABLE 4 Manufacturer recommendations for laboratory monitoring of approved EHL products

Disease	Molecule name	Brand/generic name	SPC recommendations for laboratory monitoring
Hemophilia A	rFVIII-Fc	Eloctate®/ Elocta[67]	• When using an in vitro aPTT-based one-stage clotting assay for determining FVIII activity in patients' blood samples, plasma FVIII activity results can be significantly affected by both the type of the aPTT reagent and the reference standard used in the assay.
	BAX-855	Adynovate®/ Adynovi[40]	• A field study has indicated that plasma factor VIII levels can be monitored using either a chromogenic substrate assay or a one-stage clotting assay routinely used in clinical laboratories.
	rFVIII-SC	Afstyla®[45]	• When using an in vitro aPTT-based one-stage clotting assay for determining FVIII activity in patients' blood samples, plasma FVIII activity results can be significantly affected by both the type of aPTT reagent and the reference standard used in the assay. • Also there can be significant discrepancies between assay results obtained by aPTT-based one-stage clotting assay and the chromogenic assay according to Ph. Eur. • Plasma FVIII activity in patients receiving Afstyla using either the chromogenic assay or the one-stage clotting assay should be monitored to guide the dose administered and the frequency of repeat injections. • The chromogenic assay result most accurately reflects the clinical hemostatic potential of Afstyla and is preferred. • The one-stage clotting assay result underestimates the FVIII activity level compared to the chromogenic assay result by approximately 45%. • If the one-stage clotting assay is used, multiply the result by a conversion factor of 2 to determine the patient's FVIII activity level.
	N8-GP	Esperoct®[42] (Turoctocog alfa pegol)	• The FVIII activity of Esperoct can be measured using the conventional FVIII assays, the chromogenic assay, and the one-stage assay. • When using an in vitro aPTT-based one-stage clotting assay for determining FVIII activity in patients' blood samples, plasma FVIII activity results can be significantly affected by both the type of aPTT reagent and the reference standard used in the assay. • When using a one-stage clotting assay some silica-based reagents should be avoided as they cause underestimation. • There can be significant discrepancies between assay results obtained by aPTT-based one-stage clotting assay and the chromogenic assay according to Ph. Eur.
Hemophilia B	rFIX-Fc	Alprolix®[43]	• When using an in vitro aPTT-based one-stage clotting assay for determining FIX activity in patients' blood samples, plasma FIX activity results can be significantly affected by both the type of aPTT reagent and the reference standard used in the assay. • Measurements with a one-stage clotting assay utilizing a kaolin-based aPTT reagent will likely result in an underestimation of activity level.
	rFIX-FP (CSL654)	Idelvion®[44]	• When using an in vitro aPTT-based one-stage clotting assay for determining FIX activity in patients' blood samples, plasma FIX activity results can be significantly affected by both the type of aPTT reagent and the reference standard used in the assay. • Measurement with a one-stage clotting assay using a kaolin-based aPTT reagent or Actin FS aPTT reagent will likely result in an underestimation of activity level.
	N9-GP	Refixia®[41]/ Rebinyn®	• Due to the interference of polyethylene glycol (PEG) in the one-stage clotting assay with various aPTT reagents, it is recommended to use a chromogenic assay (eg, Rox Factor IX or Biophen) when monitoring is needed. • If a chromogenic assay is not available, it is recommended to use a one-stage clotting assay with an aPTT reagent (eg, Cephascreen) qualified for use with Refixia. • For modified long-acting factor products it is known that the one-stage clotting assay results are highly dependent on the aPTT reagent and reference standard used. • For Refixia some reagents will cause underestimation (30-50%), while most silica-containing reagents will cause severe overestimation of the factor IX activity (more than 400%). Therefore, silica-based reagents should be avoided. • Use of a reference laboratory is recommended when a chromogenic assay or a qualified one-stage clotting assay is not available locally.

Abbreviations: aPTT, activated partial thromboplastin time; EHL, extended half-life product; Fc, fragment crystallizable; FIX, factor IX; FVIII, factor VIII; Ph. Eur., European Pharmacopoeia; rFIX, recombinant factor IX; rFVIII, recombinant factor VIII; SC, single chain; SPC, summary of product characteristics.

standard (if available). Laboratories should not use an assay known to give discrepant values and multiply the result by a correction factor.[13] Recommendations from the Zürich Haemophilia Forum also suggested that use of a product-specific standard might be advantageous when monitoring EHL factor concentrates.[3,12] Chromogenic assays are an appropriate method for measuring EHL-FVIII and

EHL-FIX concentrates, but performance of one-stage assays depends on the reagent used[38,47] and it would likely be impractical, and costly, for laboratories to offer multiple one-stage assays (ie, with different reagents). Of note, manufacturers are required to include recommendations for suitable test kits for laboratory monitoring of their EHL products in the summary of product characteristics (Table 4).[40-45,69]

3.3 | Influence of emicizumab on commonly used tests for hemophilia A

Emicizumab (Hemlibra®) is a chimeric bispecific monoclonal antibody that binds to both FIXa and FX, mediating the activation of FX to replace missing FVIIIa. The antibody is approved in several countries worldwide, including Europe, the United States, Japan, and Australia for prophylaxis of hemorrhage in patients with hemophilia A with or without FVIII inhibitors.[14,15] Emicizumab is not neutralized by anti-FVIII antibodies (ie, inhibitors)[70] and has a plasma half-life of 4 to 5 weeks, permitting subcutaneous administration once weekly or once every 2 or 4 weeks.[71]

Unlike FVIII, emicizumab does not require activation for cofactor activity and this has implications for the accuracy of one-stage FVIII assays and thus the selection of diagnostic assay, their modification, and interpretation of results (Table 5).72 Emicizumab and FVIII differ significantly in terms of affinity, regulation, and FIXa-enhancing activity, with the activity of emicizumab depending predominantly on the amount of FIXa generated.[15,73] Given that the amount of FIXa present in one-stage and two-stage chromogenic assays differs substantially and, moreover, due to the time-dependent difference between non-modified aPTT and chromogenic assays, the activity of emicizumab varies greatly between different diagnostic assays. Furthermore, as the molecule has a 4- to 5-week half-life, emicizumab may affect laboratory tests for up to 6 months after dosing.

FVIII activation is the rate-determining step of the global aPTT assay, and emicizumab does not require activation; therefore, patients treated with emicizumab will have a normal aPTT test result regardless of the aPTT reagent used, even at sub-therapeutic emicizumab plasma concentrations of 2-5 µg.[74] Normal-range aPTT results for patients receiving emicizumab therapy are thus misrepresentative of true coagulation status.[75] Therefore, the standard one-stage aPTT-based FVIII assay must not be used in the presence of emicizumab, as it leads to substantial overestimation of hemostatic activity.[76,77] Studies exploring the effect of emicizumab on various coagulation laboratory tests also found a strong effect of emicizumab on several other aPTT-based assays including Protein C activity, Protein S activity, and activated Protein C ratio.[76,77] Therefore, the aim of hemostatic monitoring in patients receiving emicizumab is to determine emicizumab plasma concentration.

A modified one-stage FVIII clotting assay can be used to measure plasma emicizumab concentrations.[74,79] The assay is calibrated against emicizumab using a dedicated plasma emicizumab calibrator and two levels of control,[79] which are commercially available (r^2 Diagnostics; Haemochrom Diagnostica) for use on various diagnostic platforms. Notably, the assay uses a higher sample dilution compared with the one-stage FVIII assay (1:8 sample predilution by the analyzer), enabling measurement of a dynamic range of emicizumab (10-100 µg) in the original plasma sample. Emicizumab concentrations determined with the modified one-stage assay (OSA) have been shown to correlate significantly with those determined by a non-commercial enzyme-linked immunosorbent assay (ELISA) method previously used in the HAVEN studies.[74] In a more recent analysis the modified OSA demonstrated good analytical performance for measuring emicizumab on cobas t 511/711 analyzers.80

TABLE 5 Interpretation of unexpected assay results in emicizumab-treated patients[72]

Assay	Result for patient on emicizumab
aPTT	**Overactive**—normalizes at very low concentrations of emicizumab
FVIII one-stage activity assay (aPTT-based)	**Overactive**—normalizes at very low concentrations of emicizumab
Modified FVIII one-stage assay	**Sensitive to emicizumab**—can be used to determine emicizumab plasma concentration
FVIII chromogenic activity assay (bovine components)	**Insensitive to emicizumab** (emicizumab does not accelerate FXa formation by bovine FIXa) —can be used to measure endogenous and infused FVIII levels
FVIII chromogenic activity assay (human components)	**Sensitive to emicizumab**—emicizumab and FVIIIa co-factor properties are not identical. FVIII:C is an approximation of emicizumab hemostatic activity
All Bethesda assays using one-stage FVIII methods	**False negative**—emicizumab drives coagulation via human FIX and FX in human plasma, regardless of presence of inhibitors to FVIII
Chromogenic Bethesda assay[36]	**Unaffected by emicizumab**—assay uses a bovine protein-based chromogenic assay, which is not affected by emicizumab

Abbreviations: aPTT, activated partial thromboplastin time; F, factor.

Chromogenic FVIII assays are only sensitive to emicizumab if they use human-derived factors because emicizumab drives coagulation with human FIXa and FX, but not bovine FIXa and FX.[75] Therefore, chromogenic FVIII testing using human FIXa and FX can be used for the quantitative detection of in vitro emicizumab if calibration is done versus emicizumab.[77,81] Clinical studies are under way to determine whether CSA-reported FVIII activity is sufficiently equivalent to emicizumab levels to permit clinical utility. Measurement of emicizumab in the sample may also be influenced by FVIII sample content, although pre-analytical heat treatment may potentially reduce this effect.

aPTT-based clotting assays determining FVIII activity or chromogenic FX activation assays do not reflect the combined effect of emicizumab and additional anti-hemophilic agents such as recombinant FVIII or bypassing agents such as recombinant factor VIIa (rFVIIa) or activated prothrombin complex concentrates (aPCC). FVIII activity in the presence of emicizumab can be exclusively determined using

a chromogenic FVIII assay based on bovine factors, thus permitting measurement of endogenous or infused FVIII activity independent of emicizumab.[75] Importantly, aPCC and rFVIIa should be discontinued the day before starting emicizumab treatment.[14] Use of aPCC (that contains major substrates of emicizumab[82]) should be avoided in patients receiving emicizumab prophylaxis unless no other treatment options/alternatives are available; if aPCC is indicated, the initial dose should not exceed 50 U/kg and laboratory monitoring for the diagnosis of thrombotic microangiopathy is recommended.14

Emicizumab causes false-negative results with the Bethesda assay.[37] This assay employs heat inactivation to destroy residual FVIII activity and as emicizumab is not inactivated by heat or anti-FVIII antibodies, no inhibition is detected as emicizumab restores clotting. In contrast, chromogenic Bethesda assays that use bovine components are not affected by emicizumab and can be used to measure inhibitor titer; these assays have recently been developed and are commercially available.[36-37,78] Researchers are also looking at ways to modify existing assays (ie, clot waveform analysis for evaluating FVIII activity in the presence of emicizumab) or to develop novel methods of restoring the utility of the classic Bethesda assay (ie, development of anti-idiotype antibodies to neutralize emicizumab interference).[83,84]

Although emicizumab is expected to exhibit a lower immunogenicity than FVIII,[85] the development of ADAs was reported in a small proportion of individuals during the HAVEN clinical development program, as detected by ELISA.[86] No commercial assay for direct identification of emicizumab ADAs is currently available. However, functional assays such as aPTT or the modified FVIII OSA can potentially be used to indirectly detect the possible presence of ADAs when suspected to be the cause of increased bleeding.

3.4 | Guidelines for laboratories for monitoring the use of emicizumab

There are no published recommendations regarding hemostatic monitoring during treatment with emicizumab. Table 5 summarizes current evidence on the interpretation of laboratory results in emicizumab-treated patients.14,15 FVIII chromogenic assays using human-derived components are suitable for detection of emicizumab hemostatic activity in heat-treated plasma quantitative samples.[81] However, due to mechanistic differences between emicizumab and FVIII, the aim of hemostatic monitoring in emicizumab-treated patients is to determine emicizumab plasma concentrations. A modified FVIII OSA provides an accurate method for the quantitative determination of plasma emicizumab concentration.[80] FVIII chromogenic assays that use bovine-based components can be used to measure endogenous and infused FVIII levels in the presence of emicizumab.[75] For measurement of FVIII inhibitor titer in the presence of emicizumab, a bovine plasma-based chromogenic Bethesda assay is suitable.[78] Finally, functional assays such as aPTT or the modified FVIII OSA can potentially be used to indirectly detect the presence of ADAs when suspected to be the cause of increased bleeding.

3.5 | Guidelines for clinicians for the use of EHL factor replacement and emicizumab

In the absence of standardized laboratory diagnostics clinical guidelines, or where uncertainty exists, a prudent clinical approach may be to initiate treatment with EHL factors or emicizumab using the standard dosing regimen (according to manufacturer's recommendations) to achieve low annualized bleed rates (ie, zero spontaneous bleeds) and then to de-escalate the dose or interval independently of laboratory results. Further investigation and standardization may help to establish chromogenic FVIII assays that are applicable to all EHL factors. Clinicians should also consider tailoring their EHL factor portfolio according to the available laboratory tests.

4 | CONCLUSIONS

The advent of EHL factors and emicizumab offers several advantages for the prophylactic treatment of PwH, most notably reduced treatment burden, improved patient outcomes, and treatment in the presence of FVIII inhibitors. However, the use of these agents also presents challenges in terms of patient monitoring, and existing guidelines should be updated to reflect the correct diagnostic approaches in PwH receiving these therapies. Currently, laboratories should select a chromogenic or appropriate one-stage assay that has been validated for use with the specific treatment. The standard one-stage aPTT-based FVIII assay cannot be used to measure hemostatic activity in the presence of emicizumab, as it leads to substantial overestimation of results. A modified one-stage FVIII assay provides an accurate method to monitor emicizumab in the plasma. Chromogenic FVIII testing using human FIXa and FX can quantitatively estimate in vitro emicizumab, while a bovine-based chromogenic Bethesda assay can measure inhibitor titer in the presence of emicizumab. Ultimately, increased awareness of appropriate diagnostic approaches will help to optimize patient outcomes and is an essential prerequisite to personalized therapy for PwH.

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